# DEVELOPMENT AND PRELIMINARY EVALUATION OF A DIAGNOSTIC ASSAY FOR NEUROSYPHILIS USING A REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION APPROACH

**JINNY CHARMAINE SINDLE**

Thesis submitted in fulfilment of the requirement for the degree of Master of Science (Med) in the Department of Medical Microbiology, University of Cape Town

15 August 2007

University of Cape Town

The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.

University of Cape Town

## Dedication

**To my husband, Daniël,**

**and to**

**my parents**

# DECLARATION

I, Jinny Charmaine Sindle, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise), and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

I empower the University of Cape Town to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

Signed by candidate

Signature

22/11/07

Date

# ACKNOWLEDGEMENTS

My thanks go to my supervisor, Dr Andrew Whitelaw for his help with writing the thesis, experimental design and analysing clinical results; and to Dr Lemese Ah Tow for on-the-bench assistance and guidance. My thanks go to Prof. Lafras Steyn for giving me the opportunity to research my MSc at this department, and for help on my thesis and experimental design. Thanks to Dr Nadia Wittles for help with collection of CSF samples and analysis of results.

Thanks to everyone at Medical Microbiology for their assistance in getting me started in the lab when I arrived, and for general help.

Thanks to the Bristol Myer Squibb Fund, University of Cape Town and National Health Laboratory Services Research Trust for funding and for laboratory consumables.

Finally, thank you to my friends and family, especially to my husband, Daniël and to my parents for their love, support and encouragement.

# ABSTRACT

Clinical diagnosis of neurosyphilis is difficult as it manifests non-specific symptoms which can be confused with other diseases. Laboratory diagnosis is also problematic, as an increase in mononuclear cell and protein concentrations in cerebrospinal fluid (CSF) is non-specific. A treponemal serological assay, using CSF, the CSF-fluorescent treponemal antibody-absorption (FTA-ABS) is very sensitive but has a low specificity for neurosyphilis. On the other hand, the CSF-Venereal Disease Research Laboratory (VDRL) has high specificity for neurosyphilis, but a low sensitivity.

The aim of this project was to develop a sensitive and specific diagnostic assay for neurosyphilis to detect the etiological agent of syphilis, *Treponema pallidum*, using a reverse transcription-polymerase chain reaction (RT-PCR) approach. A critical component of this assay is the inclusion of a positive control that can be used on each sample. The size of the *T. pallidum* amplicon is 366 bp and that of the positive control is 950 bp. This positive control would allow for control of the RNA extraction process as well as the RT-PCR amplification.

The positive control plasmid was constructed by ligating a 366 bp region, amplified from *T. pallidum* 16S rRNA gene, into a vector to form a phase I plasmid. PCR on the plasmid would yield a 366 bp amplicon. To distinguish between nucleic acids originating from *T. pallidum* and that of the positive control, spacer DNA was inserted into the phase I plasmid. PCR on the selected positive control plasmid yields a PCR amplicon of 950 bp. *E. coli* JM109 harbouring a positive control plasmid was used to spike CSF clinical samples.

The sensitivity of the RT-PCR reaction using *T. pallidum* RNA was optimised and the assay could detect $10^1$ *T. pallidum* equivalents of RNA. The optimal quantity of the positive control was determined to be $5 \times 10^3$ *E. coli* positive control cells, and 15 µl of eluted RNA was used in the RT-PCR reactions.

A small pilot study was performed to evaluate the RT-PCR assay, using 30 CSF samples from selected patients at the Department of Ophthalmology, Groote Schuur Hospital, Cape Town. Routine laboratory tests for neurosyphilis were performed on the CSF samples, and left over CSF was used for the RT-PCR assay. There were no false positive RT-PCR results in the 'syphilis and neurosyphilis excluded' categories. The RT-PCR assay yielded a true positive result for a definite neurosyphilis patient. In addition, the RT-PCR assay yielded one positive result out of 8 'possible neurosyphilis'

cases. It is not clear whether this patient had secondary syphilis (with central nervous system invasion by *T. pallidum*) or neurosyphilis; however as with all laboratory tests, clinical correlation should be performed. The RT-PCR negative results of the seven other 'possible neurosyphilis' patients were difficult to evaluate. It is possible that RT-PCR assay missed three samples positive for *T. pallidum* as they had features suggestive of neurosyphilis. In future, cells from CSF will be stored in an RNA stabilisation reagent, and 5, 10 and 15 µl RNA eluate will be used in the RT-PCR reaction, which may increase sensitivity of the assay.

This RT-PCR assay shows promise as a routine diagnostic assay, and may facilitate laboratory diagnosis of neurosyphilis. However, a more in-depth evaluation of the RT-PCR assay must still be performed.

# ABBREVIATIONS

A .................................................. adenosine

bDNA .......................................... branched DNA
BLAST.......................................... Basic Local Alignment Search Tool
bp ................................................ base pair(s)

C .................................................. cytidine
$Ca^{2+}$ ............................................. calcium ion
$CaCl_2$............................................. calcium chloride
CDC ............................................. Centers for Disease Control and Prevention
cDNA............................................. complementary DNA
cell(s)/ml........................................ cell(s) per millilitre
cell(s)/$mm^3$ .................................. cell(s) per cubic millimetre
cfu/ml .......................................... colony forming units per millilitre
cfu/µg .......................................... colony forming unit(s) per microgram of DNA
cm ............................................... centimetre(s)
CNS ............................................. central nervous system
CSF.............................................. cerebrospinal fluid
CTAB ........................................... cetyltrimethylammonium bromide

d................................................... day(s)
deP............................................... dephosphorylated
DEPC ........................................... diethylpyrocarbonate
DF ............................................... darkfield microscopy
DFA-TP ........................................ direct fluorescent antibody test for *Treponema pallidum*
$dH_2O$ .......................................... distilled water
DL ............................................... dephosphorylated linear
DLR.............................................. dephosphorylated linear, religated
DNA ............................................. deoxyribonucleic acid
DNase .......................................... deoxyribonuclease
dNTP............................................ deoxynucleoside triphosphate
DTT.............................................. dithiothreitol

e.g. .......... example
EDTA .......... ethylenediaminetetraacetic acid
EIA-VDRL .......... enzyme immunoassay using the VDRL antigen
ELB .......... enzymatic lysis buffer
ELISA-VDRL .......... enzyme-linked immunosorbent assay using the VDRL antigen
*et al.* .......... and others
EtBr .......... ethidium bromide
EtOH .......... ethanol

FITC .......... fluorescent isothiocyanate
FTA-ABS .......... fluorescent treponemal antibody absorption

G .......... guanosine
g/l .......... gram(s) per litre

h .......... hour(s)
$H_2O$ .......... water
HIV .......... human immunodeficiency virus

i.e. .......... that is
Ig .......... immunoglobulin
IM .......... intramuscular
IPTG .......... isopropyl-β-D-thiogalactopyranoside
IV .......... intravenous

kb .......... kilobase
kd .......... kilodalton(s)

l .......... litre(s)
LCR .......... ligase chain reaction

M .......... molar
MCB .......... Department of Molecular and Cell Biology
MCS .......... multiple cloning site
mg/dl .......... milligram(s) per decilitre
mg/ml .......... milligram(s) per millilitre

$Mg^{2+}$ ........................................ magnesium ion
$MgCl_2$ ........................................ magnesium chloride
MHA-TP ........................................ microhaemagglutination assay for *Treponema pallidum*
min ........................................ minute(s)
ml ........................................ millilitre(s)
mM ........................................ millimolar
mRNA ........................................ messenger RNA
MU ........................................ million unit(s)
MW ........................................ molecular weight

N ........................................ unspecified nucleoside
NaAc ........................................ sodium acetate
NaCl ........................................ sodium chloride
NaOH ........................................ sodium hydroxide
NASBA ........................................ nucleic acid sequence-based amplification
NCBI ........................................ National Center for Biotechnology Information
ng ........................................ nanogram(s)
ng/µl ........................................ nanogram(s) per microlitre
$NH_4OAc$ ........................................ ammonium acetate
nm ........................................ nanometre(s)

p ........................................ plasmid
PCR ........................................ polymerase chain reaction
pg ........................................ picogram(s)
pmol ........................................ picomole(s)
pmol/ml ........................................ picomole(s) per millilitre
pmol/µl ........................................ picomole(s) per microlitre

rDNA ........................................ ribosomal DNA
RE ........................................ restriction enzyme
RIT ........................................ rabbit infectivity test
RNA ........................................ ribonucleic acid
RNase ........................................ ribonuclease
rpm ........................................ revolutions per minute
RPR ........................................ rapid plasma reagin
rRNA ........................................ ribosomal RNA

RT ........................................ reverse transcription
RT-PCR ........................................ reverse transcription-polymerase chain reaction

s ........................................ second(s)
S ........................................ Svedberg unit
SAB ........................................ sample application buffer
SDA ........................................ strand displacement amplification
SDS ........................................ sodium dodecyl sulphate
SNF ........................................ supernatant fluid
subsp. ........................................ subspecies

T ........................................ thymidine
$T_a$ ........................................ annealing temperature
TAE ........................................ Tris-acetate-EDTA
TE ........................................ Tris-EDTA
$T_m$ ........................................ melting temperature
TMA ........................................ transcription-mediated amplification
TPHA ........................................ *Treponema pallidum* haemagglutination assay
TP-PA ........................................ *Treponema pallidum*-particle agglutination

U ........................................ unit(s) or enzyme unit(s)
UCT ........................................ University of Cape Town
UV ........................................ ultraviolet

V/cm ........................................ volt(s) per centimetre
v/v ........................................ volume per volume
VDRL ........................................ Venereal Disease Research Laboratories
vs. ........................................ versus

w/v ........................................ weight per volume

X-gal ........................................ 5-brom-4-chloro-3-indolyl-β-D-galactopyranoside

α ........................................ alpha
β ........................................ beta
λ ........................................ lambda
μg ........................................ microgram(s)

µg/ml ............................................ microgram(s) per millilitre
µl .................................................... microlitre(s)
µM ................................................. micromolar
ω .................................................... omega

~ ..................................................... approximately
°C ................................................... degrees Celsius
> ..................................................... greater than
% .................................................... percent

1V:1I .............................................. ratio of vector:insert of 1:1
2X YT ............................................ 2X yeast-tryptone
3' ..................................................... 3 prime
4V:1I .............................................. ratio of vector:insert of 4:1
5' ..................................................... 5 prime

# TABLE OF CONTENTS

**DEDICATION** .......... i

**DECLARATION** .......... ii

**ACKNOWLEDGEMENTS** .......... iii

**ABSTRACT** .......... iv

**ABBREVIATIONS** .......... vi

**TABLE OF CONTENTS** .......... xi

**LIST OF FIGURES** .......... xvii

**LIST OF TABLES** .......... xx

**1. CHAPTER ONE – LITERATURE REVIEW ON THE DIAGNOSIS OF SYPHILIS** .......... 1

**1.1. Introduction** .......... 1

**1.2. Epidemiology of syphilis** .......... 2

**1.3. Clinical manifestation and progress of acquired venereal syphilis** .......... 3

1.3.1. Incubating syphilis .......... 4
1.3.2. Primary syphilis .......... 5
1.3.3. Secondary syphilis .......... 5
1.3.4. Latent syphilis .......... 5
1.3.5. Tertiary syphilis .......... 6

**1.4. Symptoms of neurosyphilis** .......... 6

**1.5. Clinical manifestation and progress of congenital syphilis** .......... 8

**1.6. Treatment of syphilis** .......... 9

1.6.1. Treatment of neurosyphilis and ocular syphilis ........................................ 9

**1.7. Diagnosis of syphilis infection ........................................................ 9**

1.7.1. Microscopy and culture ................................................................ 10

1.7.1.1. Darkfield (DF) microscopy ........................................................ 10

1.7.1.2. Direct fluorescent antibody test for *Treponema pallidum* (DFA-TP) . 11

1.7.1.3. Rabbit infectivity test (RIT) ........................................................ 12

1.7.2. Serology ..................................................................................... 13

1.7.2.1. Non-treponemal serology .......................................................... 14

i. Venereal Disease Research Laboratory (VDRL) Test ........................ 14

ii. Rapid plasma reagin (RPR) test ....................................................... 15

iii. Enzyme immunoassay or Enzyme-linked immunosorbent assay using the VDRL antigen (EIA-VDRL or ELISA-VDRL) ........................................ 16

1.7.2.2. Treponemal serology ................................................................. 16

i. Fluorescent treponemal antibody absorption (FTA-ABS) .................... 17

ii. *Treponema pallidum* haemagglutination (TPHA) ............................... 17

iii. *Treponema pallidum*-particle agglutination (TP-PA) test .................... 18

1.7.3. Screening and confirmatory tests .................................................. 20

1.7.4. Molecular diagnosis ..................................................................... 22

1.7.4.1. Polymerase chain reaction (PCR) ................................................ 23

1.7.4.2. Reverse transcription-polymerase chain reaction (RT-PCR) .......... 23

**1.8. Diagnosis of neurosyphilis ................................................................ 27**

**1.9. Motivation for using RT-PCR ............................................................ 29**

**1.10. Summary ....................................................................................... 30**

**1.11. Project summary and diagnostic syphilis assay principle ................... 31**

**2. CHAPTER TWO – CONSTRUCTION OF A POSITIVE CONTROL PLASMID... ........................................................................................................ 33**

**2.1. Introduction ..................................................................................... 33**

**2.2. Materials and Methods for construction of phase I plasmid ................ 37**

2.2.1. Bacterial strains, plasmids and solutions ........................................ 37

2.2.2. Amplification of a 366 bp region from *T. pallidum* genomic DNA by PCR 38

2.2.3. Agarose gel electrophoresis ........................................................... 40

2.2.4. Purification of DNA ... 41
2.2.5. Ligation of the 366 bp fragment with the pGEM-T Easy Vector ... 41
2.2.6. Transformation ... 41
2.2.7. Phase I plasmid extractions and restriction enzyme digestion ... 43
2.2.8. DNA sequencing ... 44
2.2.9. Analysis of DNA sequences ... 44

**2.3. Results for construction of phase I plasmid ... 45**
2.3.1. PCR amplification of a region of the *T. pallidum* 16S rRNA gene ... 45
2.3.2. Cloning of the *T. pallidum* 16S rDNA internal fragment ... 46
2.3.3. Confirmation of the presence of insert in the phase I plasmid ... 48
2.3.4. Sequencing results of pTPA108 and pTPA110 ... 49

**2.4. Materials and Methods for construction of phase II plasmid ... 52**
2.4.1. Linearisation and dephosphorylation of the phase I plasmid, pTPA108 ... 52
2.4.2. Preparation of insert spacer DNA ... 53
2.4.3. Ligation of linearised pTPA108 with insert spacer DNA ... 54
2.4.4. Transformations using phase II plasmids ... 55
2.4.5. Selection of clones, plasmid extraction and restriction enzyme digestion 57
2.4.6. PCR on selected plasmids ... 57
2.4.7. Optimisation of PCR on phase II plasmids ... 57

**2.5. Results for construction of phase II plasmid ... 61**
2.5.1. Generation of spacer DNA for the construction of phase II plasmid ... 61
2.5.2. Preparation of the pTPA108 plasmid for ligation with spacer DNA ... 61
2.5.3. Cloning of the spacer DNA into phase I plasmid, pTPA108 ... 61
2.5.4. Confirmation of primer binding sites and size of insert in phase II plasmids ... 63
2.5.5. Optimisation of PCR on phase II plasmids ... 64
2.5.5.1. Results of using different parameters in PCR to eliminate non-specific bands and determine their source ... 64
2.5.5.2. Contamination ... 65

**2.6. Discussion and summary ... 65**
2.6.1. Construction and sequence analysis of phase I plasmids ... 65
2.6.2. Construction of phase II plasmids ... 67
2.6.3. PCR on phase II plasmids and non-specific products ... 68
2.6.4. Summary and conclusions ... 71

**3. CHAPTER THREE – OPTIMISATION OF THE RT-PCR ASSAY .................. 72**

**3.1. Introduction .................. 72**

**3.2. Materials and Methods .................. 73**

3.2.1. Templates, strains and CSF .................. 73
3.2.1.1. RNA templates .................. 73
3.2.1.2. Plasmid pTPA238 .................. 73
3.2.1.3. Bacterial strains .................. 74
3.2.1.4. CSF samples .................. 74
3.2.2. Optimisation of the sensitivity of the RT-PCR assay .................. 74
3.2.2.1. Baseline RT-PCR conditions .................. 74
3.2.2.2. Optimisation of the RT-PCR reaction .................. 75
3.2.3. Methods used in the evaluation of the positive control, *E. coli* TPA238 ... 79
3.2.3.1. Quantification of *E. coli* TPA238 .................. 79
3.2.3.2. RNA extractions .................. 79
3.2.3.3. Agarose gel electrophoresis .................. 79
3.2.3.4. DNase I treatment .................. 80
3.2.3.5. PCR .................. 80
3.2.4. Optimisation of preparation and use of RNA for the diagnostic RT-PCR assay .................. 80
3.2.4.1. Use of both *T. pallidum* and *E. coli* TPA238 in RT-PCR .................. 80
3.2.4.2. Preparation of RNA from simulated CSF clinical samples .................. 80
3.2.4.3. RNA precipitation tests .................. 81
i. Sodium acetate (NaAc) precipitation of RNA .................. 81
ii. Proteinase K treatment and NaAc precipitation of RNA .................. 82
iii. Ammonium acetate ($NH_4OAc$) precipitation with β-mercaptoethanol addition .................. 82
iv. Protein removal and $NH_4OAc$ precipitation of RNA .................. 82
v. Simulation of clinical samples .................. 83

**3.3. Results .................. 84**

3.3.1. Optimisation of the sensitivity of the RT-PCR reaction .................. 84
3.3.1.1. Annealing temperature .................. 84
3.3.1.2. Primer and $MgCl_2$ titrations .................. 85
3.3.1.3. dNTP and $MgCl_2$ titrations .................. 87
3.3.1.4. Various DTT concentrations .................. 88

3.3.1.5. Elongation step ........ 90
3.3.1.6. Heat denaturation of RNA prior to the RT-step ........ 91
3.3.1.7. Other optimisations ........ 92
3.3.1.8. Sensitivity of the RT-PCR assay for *T. pallidum* ........ 92
3.3.2. Evaluation of the positive control, *E. coli* TPA238 ........ 93
3.3.3. Optimisation of preparation and use of RNA from clinical samples ........ 96
3.3.3.1. Determination of the volume of *E. coli* TPA238 extracted RNA, optimal for use in the RT-PCR assay ........ 96
3.3.3.2. Optimisation of the use of *E. coli* TPA238 positive control ........ 97
3.3.3.3. RNA precipitations ........ 101
3.3.4. Specificity of the assay ........ 102

**3.4. Discussion ........ 105**
3.4.1. Optimisations of the RT-PCR reaction ........ 105
3.4.2. RNA transcription from plasmid pTPA238 ........ 106
3.4.3. Determination of the volume of extracted *E. coli* TPA238 RNA, to be used in the RT-PCR assay ........ 107
3.4.4. Amount of *E. coli* TPA238 with which to spike clinical samples ........ 107
3.4.5. RNA precipitations ........ 108
3.4.6. Specificity ........ 109
3.4.7. Summary and conclusions ........ 110

**4. CHAPTER FOUR – PRELIMINARY EVALUATION OF THE RT-PCR ASSAY ON CSF CLINICAL SAMPLES ........ 111**

**4.1. Introduction ........ 111**

**4.2. Materials and Methods ........ 111**
4.2.1. Selection of patients and storage of CSF ........ 111
4.2.2. Performing the RT-PCR assay ........ 112
4.2.3. Clinical and laboratory data collection ........ 112
4.2.4. Categorisation of clinical and laboratory data ........ 113

**4.3. Results ........ 113**
4.3.1. RT-PCR assay results ........ 113
4.3.2. Collection of clinical data ........ 115

**4.4. Analysis of results and discussion ........ 119**
4.4.1. General comments ........ 119

4.4.2. Positive results and significance in correlation with clinical information . 120
4.4.3. Negative results and significance in correlation with clinical information and suggestions for future improvements .......... 121
4.4.4. Positive control and inhibition .......... 122
4.4.5. Non-specific bands .......... 125
4.4.6. Summary and conclusions .......... 125

**5. CHAPTER FIVE – SUMMARY AND FUTURE WORK .......... 127**

**APPENDIX A. SOLUTIONS AND MEDIA .......... 130**

**APPENDIX B. METHODS .......... 134**

**APPENDIX C. MOLECULAR WEIGHT MARKERS .......... 142**

**APPENDIX D. PLASMIDS .......... 144**

**APPENDIX E. DATA COLLECTION SHEET .......... 145**

**REFERENCES .......... 146**

# LIST OF FIGURES


**Fig. 1–1.** A photomicrograph of *Treponema pallidum* .......... 2

**Fig. 1–2.** Diagrammatic representation of the natural course of syphilis .......... 4

**Fig. 1–3.** Figure graphically representing the natural course of neurosyphilis .......... 7

**Fig. 1–4.** Image of *T. pallidum* using darkfield microscopy .......... 10

**Fig. 1–5.** Image showing fluorescent *T. pallidum* organisms due to fluorescent-labelled antibodies that bind to the bacteria, in the direct fluorescent antibody test for *T. pallidum* .......... 12

**Fig. 1–6.** A positive VDRL test result .......... 15

**Fig. 1–7.** Graph depicting that the treponemal tests, FTA-ABS and MHA-TP remain reactive for life and that VDRL test results decrease with time, for untreated syphilitic patients .......... 17

**Fig. 1–8.** Diagrammatic representation of the principle of the diagnostic assay .......... 32

**Fig. 2–1.** Diagram showing the proposed positive control plasmid components .......... 34

**Fig. 2–2.** Graphical representation summarising the two major phases to produce the positive control plasmid .......... 36

**Fig. 2–3.** Diagram summarising three steps of the polymerase chain reaction and showing how exponential amplification of the target DNA occurs .......... 39

**Fig. 2–4.** Agarose-EtBr gel with PCR products obtained when using $10^9$ (lane 1), $10^7$ (lane 2), $10^5$ (lane 3) or $10^3$ (lane 4) *T. pallidum* genomic DNA equivalents .......... 45

**Fig. 2–5.** Agarose-EtBr gel showing *Eco*RI profiles of the plasmids of 10 randomly chosen white clones .......... 48

**Fig. 2–6.** Agarose (2%)-EtBr gel showing pTPA108 PCR product (lane 1), pTPA110 PCR product (lane 2) .......... 49

**Fig. 2–7.** Sequence alignment of a 366 bp portion of the published *T. pallidum* 16S rRNA gene sequence and inserts of pTPA108 and pTPA110 (sense strands) .......... 51

**Fig. 2–8.** Diagram showing the important components of the phase I and phase II plasmids .......... 52

**Fig. 2–9.** Agarose (2%)-EtBr gel, where lanes 1 – 10 depict *Eco*RI RE digested products of plasmids .......... 63

**Fig. 2–10.** PCR products using four phase II plasmids .......... 64

**Fig. 2–11.** CCA (position 546 – 548) or GCA (position 549 – 551) could have looped out and hence the sequence would be lost in the pTPA110 sequence. .. 66
**Fig. 2–12.** Illustration of the mismatch between the antisense primer and *T. pallidum* template, resulting in a nucleoside change in pTPA108 and pTPA110 plasmids. .......... 66
**Fig. 2–13.** Possible non-specific primer sites could be within the *E. coli* JM109 spacer DNA, pGEM-T Easy Vector or *T. pallidum* inserts .......... 70
**Fig. 3–1.** The combinations of RNA amounts and annealing temperatures used... 85
**Fig. 3–2.** Various primer and $MgCl_2$ concentrations were used .......... 86
**Fig. 3–3.** Varying dNTP and $MgCl_2$ concentrations are shown .......... 88
**Fig. 3–4.** Various combinations of DTT concentrations together with the RNA amounts used, are shown. .......... 89
**Fig. 3–5.** The length of elongation time at 72°C and the RNA amount used for each sample .......... 90
**Fig. 3–6.** The amounts of *T. pallidum* RNA used with or without heat denaturation of the RNA sample. .......... 91
**Fig. 3–7.** Agarose-EtBr gel showing RT-PCR products when using varying *T. pallidum* RNA amounts, as indicated in the lanes, before optimisations were performed .......... 93
**Fig. 3–8.** Agarose-EtBr gel depicting *E. coli* TPA238 RNA before (lane 1) and after (lane 2) DNase I treatment .......... 94
**Fig. 3–9.** Agarose-EtBr gel with PCR results using DNase I-treated *E. coli* TPA238 RNA .......... 95
**Fig. 3–10.** Agarose-EtBr gel with RT-PCR products obtained from two different amounts of *E. coli* TPA238 RNA .......... 95
**Fig. 3–11.** Agarose-EtBr gel showing titrations of RNA volumes used in RT-PCR.. 97
**Fig. 3–12.** RT-PCR products using RNA from $10^1$ (lanes 1 and 2), $10^2$ (lanes 3 and 4), $10^3$ (lanes 5 and 6) and $10^4$ *E. coli* TPA238 (lanes 7 and 8) .......... 98
**Fig. 3–13.** The different amounts of *E. coli* TPA238 and *T. pallidum* RNA used, in RT-PCR reactions. .......... 100
**Fig. 3–14.** Agarose-EtBr gel depicting the RT-PCR products obtained from *T. pallidum* RNA precipitated from saline (S) or CSF (C) .......... 101
**Fig. 3–15.** Lane numbers corresponding to bacterial RNA used in the RT-PCR reactions .......... 104
**Fig. 4–1.** Agarose-EtBr gel depicting RT-PCR amplicons derived from CSF samples 1 – 15 that were spiked with *E. coli* TPA238 .......... 114

**Fig. 4–2.** Agarose-EtBr gel showing RT-PCR products using RNA extracted from samples 4 and 14 .......... 115

# LIST OF TABLES

**Table 1–1.** Comparative sensitivities of various direct methods used to diagnose infection with *T. pallidum* ..... 13
**Table 1–2.** Sensitivity and specificity of various non-treponemal and treponemal serological tests, at different stages of untreated syphilis infection ..... 19
**Table 1–3.** Table showing which tests are appropriate for the different stages of syphilis, according to the different sensitivities and specificities of the diagnostic tests at different stages of syphilis ..... 21
**Table 1–4.** Table comparing the sensitivities and specificities and various other parameters of PCR and RT-PCR assays performed on *T. pallidum* samples ..... 24
**Table 1–5.** Differential diagnoses of neurosyphilis ..... 27
**Table 2–1.** Bacterial strains and plasmids used in construction of a phase I plasmid. ..... 37
**Table 2–2.** Competent *E. coli* JM109 transformed with samples, in preparation of phase I plasmids. ..... 43
**Table 2–3.** Transformation results in preparation of the phase I plasmid. ..... 47
**Table 2–4.** Samples were transformed into competent *E. coli* JM109 cells, in preparation of phase II plasmids ..... 56
**Table 2–5.** PCR parameters were varied in PCR reactions in order to eliminate the formation of non-specific products ..... 59
**Table 2–6.** These transformations were performed in preparation of a phase II plasmid. ..... 62
**Table 3–1.** Table showing the experiments performed to optimise the sensitivity of the RT-PCR assay, using *T. pallidum* RNA as template ..... 77
**Table 3–2.** Table showing bacteria from which RNA was extracted, and reasoning why they were chosen in the specificity of the assay. ..... 102
**Table 4–1.** Demographic information, diagnosis, laboratory results and treatment outcome of patients, compared to the RT-PCR assay result. ..... 116
**Table 4–2.** Depiction of the number of RT-PCR positive results in comparison to categories 1 – 4 ..... 120
**Table 4–3.** Comparison of laboratory tests performed on patients' CSF and inhibition of the RT-PCR assay. ..... 124

# 1. CHAPTER ONE – LITERATURE REVIEW ON THE DIAGNOSIS OF SYPHILIS

## 1.1. INTRODUCTION

There are two main theories about the origin of syphilis.

According to the New World or Columbian theory, syphilis was present in the area now known as Haiti and was transferred to Europe by Columbus in the 1400s. The second theory, the pre-Columbian or Old World theory states that syphilis started in central Africa and was carried to Europe before Columbus' journey (Singh & Ramonowski, 1999). However, by 1495 syphilis had spread throughout Europe, after which it spread to India (1498) and China (1505).

Various names for syphilis have been coined. Due to embarrassment, the English and Germans called syphilis "the French pox", Russians called it "the Polish sickness", Poles, "the German sickness", Japanese, "the Canton rash" or "the Chinese ulcer", etc (Heymann, 2006). However, the name that remained was 'syphilis'. In 1530, Hieronymus Fracastorius coined the word 'syphilis', which came from a shepherd's name, Syphilus in his poem 'Syphilis is the French Disease'.

Other events in syphilis history, includes that it was first believed that gonorrhoea and syphilis were the same disease. Ricord proved this false; and also characterised syphilis into primary, secondary and tertiary phases (Singh & Romanowski, 1999).

It was demonstrated in 1905 that the bacterium, *Treponema pallidum* subsp. *pallidum* is the etiological agent of the disease, syphilis (reviewed in Singh & Romanowski, 1999; Wicher *et al.*, 1999; LaFond & Lukehart, 2006). *Treponema pallidum* subsp. *pallidum* belongs to the order *Spirochaetales*, family *Spirochaetaceae* and genus *Treponema*. The organism was named *Treponema* because of its spiralled, twisted thread appearance and *pallidum* due to its pale colour (Fig. 1–1).

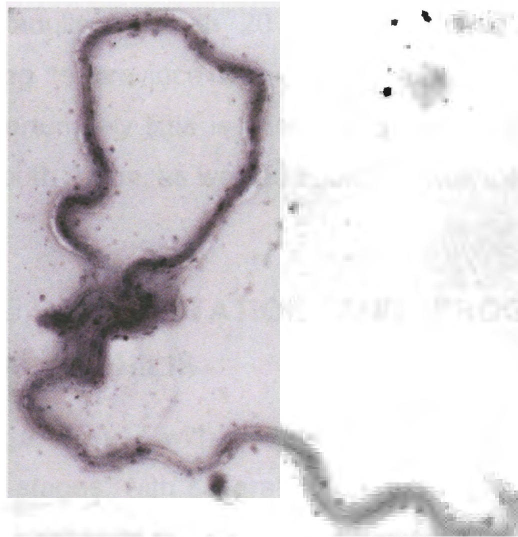


**Fig. 1–1.** A photomicrograph of *Treponema pallidum* (Centers for Disease Control and Prevention, and VDRL department). Note its thin, spiralled twisted shape.

It is a motile Gram-negative bacterium that has endoflagella, aiding in its corkscrew movement. Other pathogenic treponemes that cause yaws; pinta; and bejel, non-venereal or endemic syphilis, respectively, are *Treponema pallidum* subsp. *pertenue*, *Treponema carateum* and *Treponema pallidum* subsp. *endemicum*. *T. pallidum* subsp. *pallidum* will be referred to as *T. pallidum* in this thesis.

## 1.2. EPIDEMIOLOGY OF SYPHILIS

The two major routes of acquisition of syphilis are by sexual contact (horizontal) or vertically from mother to foetus. For horizontally-acquired syphilis, *T. pallidum* is transferred mainly by sexual contact, but minor routes of infection include kissing; blood transfusion; and accidental inoculation by needle stick or working with infected clinical material (e.g. syphilis of fingers is most predominant in medical personnel). (Tramont, 2000).

The prevalence of reported primary and secondary syphilis cases in the United States of America (USA), from 2004 to 2005, increased by 9.3% from 7 980 to 8 724. Early latent cases increased by 5.3% (7 768 – 8 176). The number of cases of late latent and late syphilis decreased by 7.2% (from 17 300 to 16 049). The number of cases of all stages of syphilis decreased by 0.4% (from 33 419 to 33 278) (Department of Health and Human Services, 2006).

Interestingly, in South Africa, the reported antenatal syphilis cases decreased from 3.2% (2002) to 2.7% (2003) to 1.6% (2004). The highest prevalence of syphilis of

1.8% was among adults between 20 – 24 years old. Additionally, the highest prevalence according to province was in the Northern Cape (7%) (Department of Health, 2005). Unfortunately little is known about the number of cases of latent and tertiary syphilis in South Africa, as well as about prevalence of syphilis in males.

## 1.3. CLINICAL MANIFESTATION AND PROGRESS OF ACQUIRED VENEREAL SYPHILIS

Venereal syphilis is a disease with a multitude of clinical manifestations and can be divided into several stages, with several different symptoms at most stages (Singh & Romanowski, 1999; Wicher *et al.*, 1999; Goldmeier & Guallar, 2003) (Fig. 1–2). Given the diverse range of symptoms and many non-specific symptoms of syphilis, it seems appropriate that Sir William Osler said "He who knows syphilis, knows medicine". The disease is normally not life-threatening at the early infectious stages. However, it can become fatal later on.

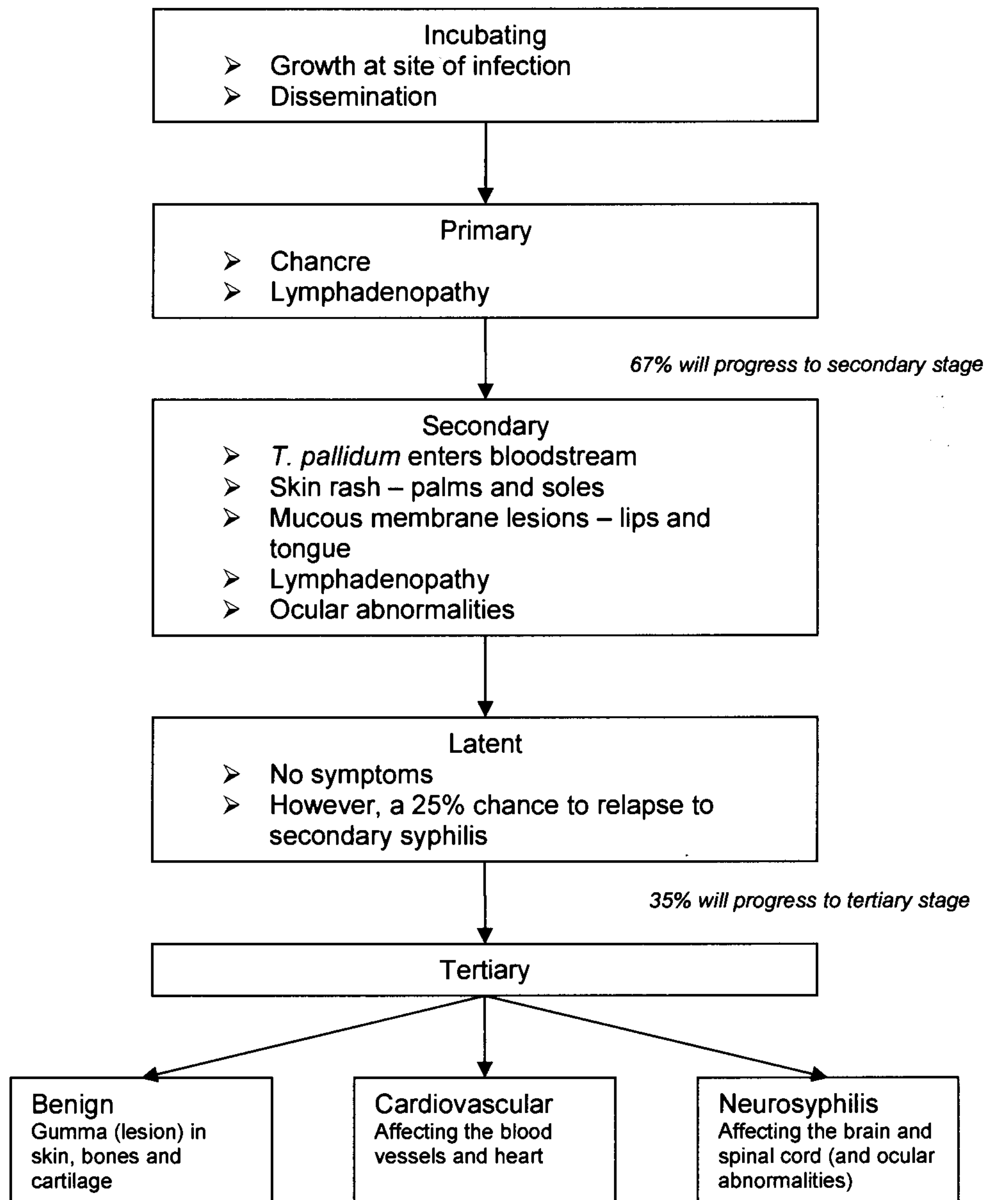



**Fig. 1–2.** Diagrammatic representation of the natural course of syphilis (Margo & Hamed, 1992; Singh & Romanowski, 1999; Wicher *et al.*, 1999; Tramont, 2000; LaFond & Lukehart, 2006).

### 1.3.1. Incubating syphilis

*T. pallidum* enters the intact mucous membrane or abraded skin, and grows at the site of infection (Tramont, 2000; LaFond & Lukehart, 2006). The organism enters the lymphatics and blood stream, and disseminates throughout the body. *T. pallidum* divides every 30 – 33 hours (h).

### 1.3.2. Primary syphilis

Approximately 3 weeks (range 3 – 90 days [d] ) after infection, a painless red indurated lesion or ulcer, called a chancre, is formed at the site of infection e.g. external genitalia and anus (Singh & Romanowski, 1999; Wicher *et* al., 1999; Tramont, 2000). In some cases, atypical lesions form and sometimes a lesion does not form. Painless regional lymphadenopathy can also occur. The primary stage is infectious. Without treatment, the chancre heals in about 3 – 6 weeks (range 1 – 12 weeks) (Tramont, 2000).

### 1.3.3. Secondary syphilis

The number of *T. pallidum* organisms is at the maximum in the infected host during the secondary phase of syphilis, particularly in the blood (Margo & Hamed, 1992; Singh & Romanowski, 1999; Wicher *et al.*, 1999; Tramont, 2000). Approximately 2 – 8 weeks after appearance of the chancre, symptoms of the secondary phase begin (Tramont, 2000). Maculopapular skin lesions or rash, especially on the palms and soles may form, as well as mucous membrane lesions (e.g. on the lips and tongue). Condylomata lata, which are lesions found in moist areas e.g. genitalia and anus, and lymphadenopathy can also occur. Other features include weight loss, sore throat, myalgia, headache, fever, asymptomatic meningitis and alopecia. This stage is highly infectious. Without treatment symptoms disappear in 3 – 12 weeks. A primary chancre may still be present during the secondary phase (Tramont, 2000).

### 1.3.4. Latent syphilis

Latent disease is divided into early and late latent diseases, which are both asymptomatic (Singh & Romanowski, 1999; Wicher *et al.*, 1999).

Latent disease is divided into two phases, as within one year (or less) after infection patients have a significant chance of relapse (~ 25%), (Singh & Romanowski, 1999) to the symptoms of secondary syphilis, and thus becoming infectious again (early latent). After one year, the chance of relapsing is much lower (late latent). Definitions of early latent syphilis thus vary, with some regarding it as occurring within one year of infection, while others take two years post infection as the dividing point between early and late latent syphilis (Singh & Romanowski, 1999; Wicher *et al.*, 1999). During late latent phase, patients are considered non-infectious to their sexual partner (Wicher *et al.*, 1999; LaFond & Lukehart, 2006). The mother can, however, pass *T. pallidum* to the foetus, which may result in congenital syphilis (section 1.5).

### 1.3.5. Tertiary syphilis

This occurs several years after infection, and patients are usually non-infectious during this stage (Singh & Romanowski, 1999; Wicher *et al.*, 1999). It was observed that approximately 35% of untreated patients enter tertiary syphilis, in studies performed between 1890 and 1910 (Wicher *et al.*, 1999).

In investigations performed in the pre-penicillin era, it was estimated that benign syphilis affected 15% of patients; cardiovascular syphilis affected 10 – 25% of patients; neurosyphilis affected 10% of patients, and approximately 60% may be unharmed (Wicher *et al.*, 1999). Benign syphilis normally involves non-vital structures such as skin, bones, cartilage and certain organs. Manifestations of cardiovascular syphilis include aortic aneurysm, aortic regurgitation and coronary artery and ostial stenosis (Singh & Romanowski, 1999). Neurosyphilis affects the brain and central nervous system (sections 1.4 and 1.8).

## 1.4. SYMPTOMS OF NEUROSYPHILIS

Neurosyphilis is a complex disease, with overlap between the phases, and with many non-specific symptoms. It can occur in the secondary and tertiary phases. Neurosyphilis affects the central nervous system (CNS), consisting of the brain and spinal cord (Singh & Romanowski, 1999; Tramont, 2000). In a study by Lukehart *et al.* (1988), *T. pallidum* was isolated from CSF of 30% of patients with untreated primary and secondary syphilis. This suggests that invasion of the CNS by *T. pallidum* is common in early syphilis.

After first invasion of CSF by *T. pallidum*, untreated individuals may follow one of various courses (Fig. 1–3):

- Asymptomatic neurosyphilis
- Acute syphilitic meningitis
- Meningovascular neurosyphilis
- Parenchymatous neurosyphilis

Early asymptomatic neurosyphilis is when no neurological symptoms occur, but CSF has one or more abnormalities: pleocytosis, a higher protein concentration, decreased glucose concentration, or a positive non-treponemal test (Tramont, 2000). Asymptomatic neurosyphilis can spontaneously resolve. Alternatively, acute syphilitic meningitis can occur, with symptoms including headache, meningeal irritation, confusion and ocular abnormalities e.g. optic neuritis, uveitis and iritis.

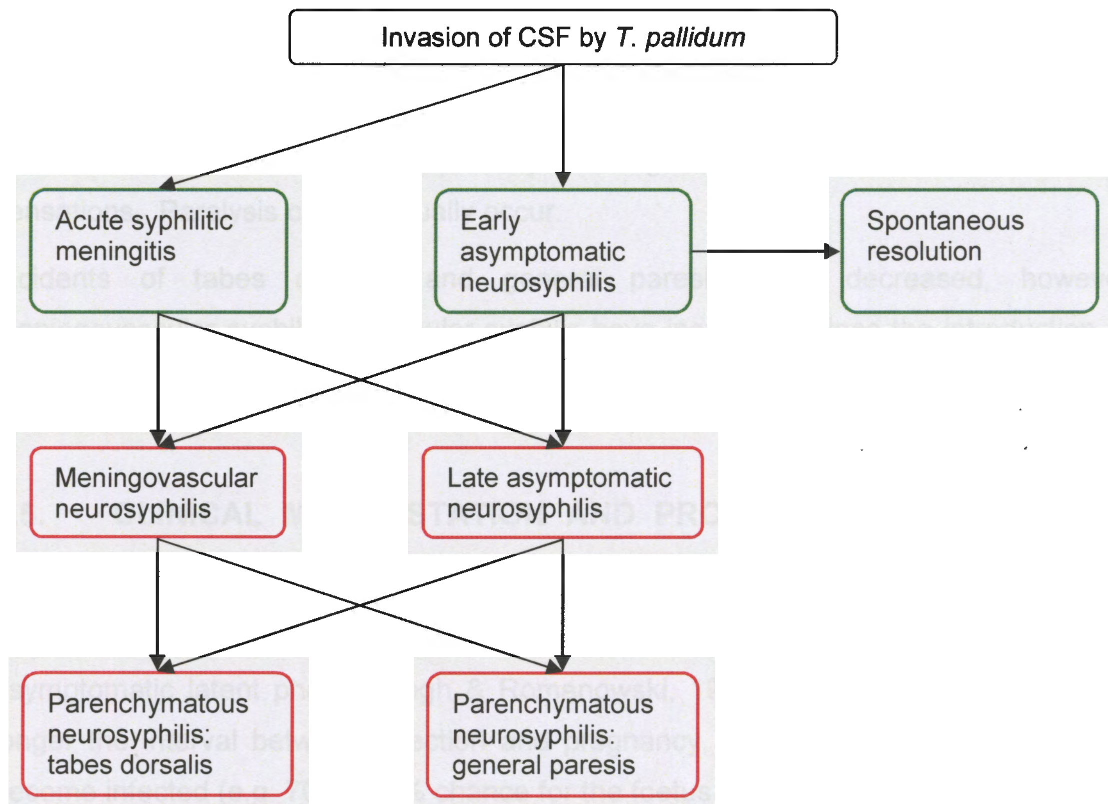



**Fig. 1–3.** Figure graphically representing the natural course of neurosyphilis. Adapted from Tramont, 2000. The green blocks indicate neurosyphilis during early or tertiary syphilis; and the red blocks indicate neurosyphilis during tertiary syphilis.

The disease may then remain asymptomatic or develop to meningovascular syphilis and/or parenchymatous neurosyphilis. Meningovascular neurosyphilis affects the small blood vessels of the meninges, brain and spinal cord, and symptoms include seizure, headaches, encephalitic presentation and cranial nerve palsies (Singh & Romanowski, 1999; Tramont, 2000).

Parenchymatous neurosyphilis includes general paresis (involvement of cerebral cortex) and tabes dorsalis (involvement of spinal cord). The word paresis in the context of syphilis is also an acronym describing some of the common clinical features (Tramont, 2000):

**P**ersonality (emotional lability, paranoia);
**A**ffect (carelessness in appearance);
**R**eflexes (hyperactive);
**E**ye (Argyll Robertson pupils);
**S**ensorium (illusions, delusions);
**I**ntellect (decreased memory, judgement and insight); and

Speech (slurred speech).

Tabes dorsalis involves the spinal cord, where damage of the spinal cord and peripheral nervous tissue occur which causes decreased muscle function. Symptoms include loss of co-ordination, difficulty walking, wide-based gait and abnormal sensations. Paralysis can eventually occur.

Incidents of tabes dorsalis and general paresis have decreased, however meningovascular syphilis and ocular syphilis have increased, since the introduction of antibiotics (Margo & Hamed, 1992). The reason for this shift is unknown.

## 1.5. CLINICAL MANIFESTATION AND PROGRESS OF CONGENITAL SYPHILIS

*T. pallidum* can be transferred from mother to foetus, even when the mother is in the asymptomatic latent phase (Singh & Romanowski, 1999; Aldave *et al.*, 2001). The longer the interval between infection and pregnancy, the less likely the foetus is to become infected (e.g. 70 – 100% chance for the foetus to be infected if the mother is in the primary stage, 40% for early latent syphilis and 10% for late latent disease). Untreated syphilis of the mother can affect pregnancy, for example, causing: spontaneous abortion, stillbirth, premature delivery or perinatal death. Congenital syphilis is divided into early and late stages, in which the late stage occurs more than two years after birth.

Early congenital syphilis includes symptoms such as mucocutaneous lesions, anaemia, neurosyphilis, periostitis and osteochondritis, and corresponds to the secondary stage of sexually transmitted syphilis. Uveitis is the most common ocular complication of early congenital syphilis (Aldave *et al.*, 2001; Chakraborty & Luck, 2007).

Late congenital syphilis can manifest with lymphadenopathy, condylomata, anaemia, neurosyphilis and bone lesions, and corresponds to the secondary and tertiary stages of acquired syphilis. Ocular complications are common in congenital syphilis. The most common ocular manifestation of late congenital syphilis is interstitial keratitis (Singh & Ramonowski, 1999; Aldave *et al.*, 2001).

Adults who had congenital syphilis can become re-infected with *T. pallidum* (Margo & Hamed, 1992).

## 1.6. TREATMENT OF SYPHILIS

Before the penicillin era, treatment of syphilis included organic arsenical compounds, mercury and bismuth. Mahoney *et al.* successfully treated syphilis with penicillin in 1943 (Singh & Romanowski, 1999). Nowadays, early (primary, secondary and early latent) and late syphilis (late latent and tertiary) are still treated using benzathine benzyl penicillin. Early syphilis is treated with 2.4 million units (MU) intramuscularly, once; and late syphilis with 2.4 MU intramuscularly weekly, for three weeks (Singh & Romanowski, 1999). If patients are hypersensitive to penicillin, alternatives (available only for some stages of syphilis) can be administered, or patients can be desensitised to penicillin (Margo & Hamed, 1992).

After effective initial treatment with antibiotics, especially penicillin, the Jarisch-Herxheimer reaction often occurs (Tramont, 2000). The symptoms include fever, chills, headache and myalgia. It occurs for approximately 12 to 24 h, and mostly in secondary syphilis, and is usually treated with aspirin.

### 1.6.1. Treatment of neurosyphilis and ocular syphilis

Patients should be treated with 18 to 24 MU, of aqueous penicillin G daily, given intravenously for 10 to 14 d; or 2.4 MU daily of procaine penicillin G given intramuscularly with 500 mg oral probenecid daily, for 10 to 14 d (Singh & Romanowski, 1999). Probenecid is administered to increase penicillin levels in serum and CSF (Singh & Romanowski, 1999; Aldave *et al.*, 2001). Benzathine benzyl penicillin is not recommended as it does not reach high enough levels in CSF (Singh & Romanowski, 1999).

This study is aimed at developing a diagnostic assay for neurosyphilis, and the emphasis will thus be placed on the diagnosis of neurosyphilis, specifically molecular diagnosis of neurosyphilis.

## 1.7. DIAGNOSIS OF SYPHILIS INFECTION

The clinical diagnosis of syphilis is not always easy (Margo & Hamed, 1992; Wicher *et al.*, 1999). In early primary syphilis, for example, lesions may not be seen due to their position (e.g. rectum, cervix); or may not have developed. In addition, genital ulcers can be caused by other organisms, e.g. *Haemophilus ducreyi* and herpes simplex

virus. Differential diagnosis of the primary chancre includes balanitis, trauma, erysipelas, genital herpes, and chancroid.

Secondary syphilis can manifest clinically in a wide variety of ways. Some of the symptoms are completely non-specific (such as fever, headache, lymphadenopathy), while the more characteristic features of secondary syphilis such as the rash can still be confused with other conditions such as viral exanthems. Differential diagnosis of secondary syphilis includes conditions as diverse as pityriasis rosea, rosacea, erythema multiforme, and psoriasis (Wicher *et al.*, 1999).

The latent stage of syphilis is asymptomatic, and would only be suspected if a careful clinical history is taken, or else be found incidentally by serological screening of the patient.

It is clear from the above description that syphilis can mimic a number of different conditions and for this reason it has been called 'The Great Imitator' (Tramont, 2000). It is almost impossible to confirm a diagnosis on clinical grounds alone, and laboratory tests thus form a critical component of the investigation of suspected cases of syphilis. Laboratory tests include microscopy, culture, serology and molecular diagnosis.

### 1.7.1. Microscopy and culture

#### *1.7.1.1.* *Darkfield (DF) microscopy*

Due to the narrowness of *T. pallidum*, it cannot be viewed by the conventional light microscope but can be viewed using DF microscopy (Fig. 1–4). *T. pallidum* present in exudates from lesions can be recognised by their distinct shape and motility, for example, the number of coils, length, width, wavelength, wave depth and rotation which are slightly different to other *Treponema* species (Larsen *et al.*, 1995).

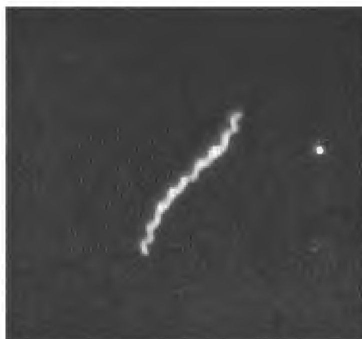


**Fig. 1–4.** Image of *T. pallidum* using darkfield microscopy (Centers for Disease Control and Prevention, and Schwartz).

Once the exudate is collected, work must be done quickly so that the bacteria are still alive when viewed under the microscope. The serous fluid from the lesions should ideally be free of erythrocytes, other organisms and tissue remains. If these are present, *T. pallidum* may be hidden, contort or bend in a way that its shape and motility cannot be recognised.

Light for DF microscopy comes from light rays that strike the object in the field obliquely, and the rays reflected from the object enter the microscope (no direct rays enter the microscope). Hence the object looks illuminated, against a dark background (Larsen *et al.*, 1995; Wicher *et al.*, 1999; Clyne & Jerrard, 2000).

DF is not very sensitive (73.8 – 78.8%), and *T. pallidum* can be confused with other non-pathogenic spirochetes that reside in the mouth and anal area, therefore DF should not be used for examining exudates from those areas (Table 1–1). DF is technically difficult and not widely available. However, this method is useful in the diagnosis of early primary syphilis when antibodies are not yet present (Wicher *et al.*, 1999).

#### *1.7.1.2. Direct fluorescent antibody test for* Treponema pallidum *(DFA-TP)*

In this method, antibody labelled with fluorescent isothiocyanate (FITC) can be used to detect *T. pallidum* present in exudate from lesions (Larsen *et al.*, 1995; Wicher *et al.*, 1999). The antibody preparation is absorbed with non-pathogenic treponemes to render them specific to pathogenic treponemes. However, the antibodies cannot distinguish between the pathogenic treponemes, *T. pallidum* subsp. *pertenue*, *T. pallidum* subsp. *endemicum*, *T. carateum* and *T. pallidum* subsp. *pallidum.* Some studies suggest that when using monoclonal antibodies versus polyclonal antibodies in the DFA-TP, the test is more specific (Hook *et al.*, 1985; Romanowski *et al.*, 1987). Using monoclonal antibodies in DFA-TP is as sensitive or more sensitive than DF (Hook *et al.*, 1985; Cummings *et al.*, 1996). If *T. pallidum* is present, FITC-labelled antibody will bind and the test is read as positive when fluorescent treponemes with their typical morphology, are seen using the microscope (Larsen *et al.*, 1995) (Fig. 1–5). DFA-TP should be used instead of DF, when examining exudates from the mouth and anal regions. Problems with DFA-TP are that it is technically difficult, a fluorescence microscope with darkfield condenser is needed and a negative result does not exclude syphilis as various technical factors influence the detection of *T. pallidum* (Larsen *et al.*, 1995; Cummings *et al.*, 1996). As with darkfield microscopy, DFA-TP is also not very sensitive (73 – 100%) (Table 1–1).

**Fig. 1–5.** Image showing fluorescent *T. pallidum* organisms due to fluorescent-labelled antibodies that bind to the bacteria, in the direct fluorescent antibody test for *T. pallidum* (Centers for Disease Control and Prevention).

#### *1.7.1.3. Rabbit infectivity test (RIT)*

*T. pallidum* cannot be cultured on artificial media in the laboratory, but can be cultured in rabbits, mainly in rabbit testes (Wicher *et al.*, 1999). Other animals could be used, but rabbits are ideal: a lesion is formed at the site of inoculation; *T. pallidum* can be transferred from one animal to the next by using minced lymph nodes or testes; and if the rabbit has syphilis, serological tests for syphilis become reactive (Larsen *et al.*, 1995).

An example of how RIT works is as follows (Lukehart *et al.*, 1988): A sample (CSF or any other specimen) is collected from a patient and inoculated into a rabbit, usually in the testicles or under the skin (Larsen *et al.*, 1995). The sample must be less than 1 h old; or flash frozen straight after collection and kept at temperatures below –80°C as *T. pallidum* needs to be viable.

The rabbits are examined regularly for three months for orchitis (evidence of infection by *T. pallidum*), by DF microscopy of testicular extracts, and serologically, by the Venereal Disease Research Laboratories (VDRL) and the fluorescent treponemal antibody absorption (FTA-ABS) tests (section 1.7.2). If seroconversion occurs, animals are sacrificed and lymph nodes and testicular tissue are minced and injected intratesticularly into a second rabbit. Generally, confirmation of infection in a second rabbit is required to confirm the presence of *T. pallidum* in a patient's sample.

Rabbits with no evidence of infection by the end of three months are said to be uninfected (Lukehart *et al.*, 1988). RIT is highly sensitive, although rare results of false negatives do occur. However, a positive result is conclusive evidence.

Disadvantages of the RIT are that it cannot be routinely used in clinics due to the need for rabbits; lengthy diagnosis process, as the incubation time after inoculation lasts weeks to months thus making clinical management difficult; variation in susceptibility by rabbits to infection; and expense (Wicher *et al.*, 1999). However, a positive aspect is its high sensitivity and specificity (Table 1–1) (Lukehart *et al.*, 1988; Wicher *et al.*, 1999).

**Table 1–1.** Comparative sensitivities of various direct methods used to diagnose infection with *T. pallidum* (Turner *et al.*, 1969; Ramonowski *et al.*, 1987; Larsen *et al.*, 1995; Centurion-Lara *et al.*, 1997; Wicher *et al.*, 1999).

| Test | Sensitivity (%) |
|---|---|
| DF | 73.8 – 78.8 |
| DFA-TP | 73 – 100 |
| RIT | ~ 100 |

### 1.7.2. Serology

Due to the problems experienced with microscopy and culture, serological tests have been developed. Serology is currently the mainstay of syphilis diagnosis. Serological tests essentially are a way of detecting the presence of patient's antibodies by interactions with an antigen-mixture, *in vitro*. In this way, the presence of *T. pallidum* can be indirectly determined by the presence of these antibodies by serology (Wicher *et al.*, 1999).

Two types of antibodies are formed during infection with treponemes, namely non-treponemal and treponemal antibodies (Wicher *et al.*, 1999). Treponemal and non-treponemal antibodies appear at similar times. It is not known which appears first, although treponemal antibodies are the first to be detected after infection, by laboratory testing (Wicher *et al.*, 1999). Non-treponemal antibodies are directed against lipoidal material and possibly cardiolipin released from treponemes. The lipoidal material is possibly derived from *T. pallidum* itself; or from the interaction of host tissues with *T. pallidum* (lipids derived from host) and are incorporated into the membrane of the metabolically limited *T. pallidum* (Larsen *et al.*, 1995; Tramont, 2000; LaFond & Lukehart, 2006). In addition to being formed during treponemal diseases, other

disorders can also trigger production of non-treponemal antibodies because of damaged tissue. Examples of these disorders are autoimmune diseases, a variety of acute bacterial and viral infections, chronic inflammatory conditions and patients with narcotic addictions, when using injections (Young, 1998; Wicher *et al.*, 1999; Clyne & Jerrard, 2000).

The first serological test, the complement fixation test, used to diagnose syphilis infection was reported in 1906 by Wassermann *et al.* (reviewed in Wicher *et al.*, 1999). The complement fixation and precipitation tests were standard laboratory tests for syphilis for several decades. The current standard non-treponemal tests for syphilis are the Venereal Disease Research Laboratories (VDRL) and rapid plasma reagin (RPR) tests; and the current standard treponemal test at Groote Schuur Hospital, Cape Town, South Africa is the fluorescent treponemal antibody absorption (FTA-ABS) test. However other tests are in use in different diagnostic laboratories. The following are some examples of various diagnostic tests that have been developed.

#### *1.7.2.1. Non-treponemal serology*

Non-treponemal serology detects the presence or absence of non-treponemal antibodies. It is called 'non-treponemal' as the antigen used in the non-treponemal tests is not unique to *T. pallidum* (LaFond & Lukehart, 2006).

##### *i. Venereal Disease Research Laboratory (VDRL) Test*

The VDRL test was developed in 1946 and includes cardiolipin, lecithin and cholesterol as antigen. Cardiolipin, a phospholipid present in all mammalian cells, specifically mitochondria, was originally isolated from beef heart in 1941. (Beef heart is rich in mitochondria.) It was found that serum of patients with syphilis contained non-treponemal immunoglobulin (Ig) M and IgG antibodies against cardiolipin. Non-treponemal antibodies can also be called phospholipid, VDRL or cardiolipin antibodies.

In the VDRL test, heated serum (heated to enhance reactivity) and then the VDRL antigen are placed on a glass slide, and rotated mechanically (Wicher *et al.*, 1999; Clyne & Jerrard, 2000). When the antibodies from the test serum combine with the VDRL antigen, antigen-antibody complexes form flocculates, which when viewed using a microscope indicate a positive result (Fig. 1–6). If no flocculation is seen, this is taken as a negative result.

**Fig. 1–6.** A positive VDRL test result. Note the flocculates indicating a positive result where antibodies from test serum have combined with VDRL antigen (Centers for Disease Control and Prevention, and Renelle Woodall).

The VDRL antigen is also called reagin antigen, and consists of, most importantly: cardiolipin (0.03%), lecithin (0.21%) and cholesterol (0.9%). Lecithin and cholesterol are added because antibodies are unable to bind cardiolipin on its own, as it is a hapten; and addition of lecithin and cholesterol increases the sensitivity of the test.

VDRL is the test most often used for examination of cerebrospinal fluid (CSF) in patients suspected to have neurosyphilis (section 1.8).

### *ii. Rapid plasma reagin (RPR) test*

The RPR test is a modification of the VDRL test, in which the VDRL antigen is also used, but the test is easier to perform and to read (Wicher *et al.*, 1999; Clyne & Jerrard, 2000). Unheated serum is added to the reagin antigen on a plastic card, and rotated mechanically. Flocculation occurs if phospholipid antibodies are present. The antigen mixture also contains charcoal particles that become trapped in the flocculation (antibody-antigen aggregates) to enhance visualisation of flocculation; therefore flocculation can be seen without using a microscope.

The VDRL and RPR can be negative during early primary syphilis, for example due to the antibodies not yet being detectable. Compared to the VDRL test, RPR is easier to perform and more sensitive especially in primary syphilis. The sensitivities of VDRL and RPR during primary syphilis are 74 – 87% and 77 – 99%, respectively (Table 1–2).

VDRL and RPR are both qualitative and quantitative tests. In the qualitative test, undiluted serum is used to detect the presence or absence of antibodies.

In the quantitative test, serial two-fold dilutions of serum are performed, until an endpoint is reached. Quantitative test results are reported as the endpoint titre, which

is the highest dilution to give a positive result. For example, the highest dilution '1:8' is reported as '8'. Quantitative results are useful to monitor treatment (Larsen *et al.*, 1995). A fourfold or greater decrease in titre (e.g. from 32 to 8) after six months implies a high probability of successful treatment, and decreasing amount of antibodies (Fig. 1–7).

False negative results can be read when performing the VDRL and RPR tests, called the prozone effect (Larsen *et al.*, 1995; Young, 1998; Clyne & Jerrard, 2000). This occurs because of saturation of antigenic sites by excess antibodies, which prevents agglutination. This prozone effect does not occur often (1 – 2% of patients with secondary syphilis). It can be corrected by diluting the serum in two-fold steps e.g. 1:2 to 1:4 to 1:8 etc, which eliminates the problem of excess antibodies.

#### *iii. Enzyme immunoassay or Enzyme-linked immunosorbent assay using the VDRL antigen (EIA-VDRL or ELISA-VDRL)*

This process also allows determination of the presence of phospholipid antibody in test serum, using the VDRL antigen. The principle of this technique is that both IgM and IgG antibodies in the serum bind to the VDRL antigen, which is fixed onto microtitre plates (Pedersen *et al.*, 1987; Larsen *et al.*, 1995; Wicher *et al.*, 1999). Enzyme-labelled antiglobulin antibody then binds to the serum antibody. A substrate (chromogen) for the enzyme is added and the reaction between the enzyme-antibody and substrate causes a coloured product, the intensity of which is determined spectrophotometrically. The intensity of colour is proportional to the amount of antibodies in the serum. The sensitivity of EIA-VDRL during primary syphilis is 93% (Table 1–2).

#### *1.7.2.2. Treponemal serology*

These tests detect treponemal antibodies from sample directed against treponemal antigens, usually in the form of *T. pallidum* antigen fixed to a plate, cells or latex particles. A disadvantage is that treponemal antibodies cannot distinguish between *T. pallidum* and other pathogenic and non-pathogenic treponemes (Wicher *et al.*, 1999), if the serum or plasma is not passed through the appropriate treponemal culture filtrate.

### *i. Fluorescent treponemal antibody absorption (FTA-ABS)*

This is the most widely used treponemal test (Larsen *et al.*, 1995; Wicher *et al.*, 1999; Clyne & Jerrard, 2000). Test serum is diluted 1:5 in sorbent, which is a culture filtrate of non-pathogenic *T. phagedenis* Reiter, to remove treponemal antibodies that may be produced against non-pathogenic treponemes. The serum is then layered over a glass slide affixed with *T. pallidum* antigen. After a few washes, fluorescent-labelled antibodies to IgG are added and the slide washed again. The intensity of fluorescence is read using UV microscopy.

The sensitivity of FTA-ABS for primary syphilis is 70 – 100% and for secondary, latent and tertiary syphilis is 96 – 100% (Table 1–2). FTA-ABS may not be reliable in early primary syphilis as treponemal antibodies may not yet be detectable.

FTA-ABS is not a quantitative test (Clyne & Jerrard, 2000). FTA-ABS remains reactive for life in most patients, therefore it cannot be used to monitor response to therapy; nor does it give an indication of active disease (Fig. 1–7). It does give an indication of development of immunity. On the other hand, non-treponemal serology detects active disease, so can be used to evaluate treatment (quantitative); but not development of immunity.

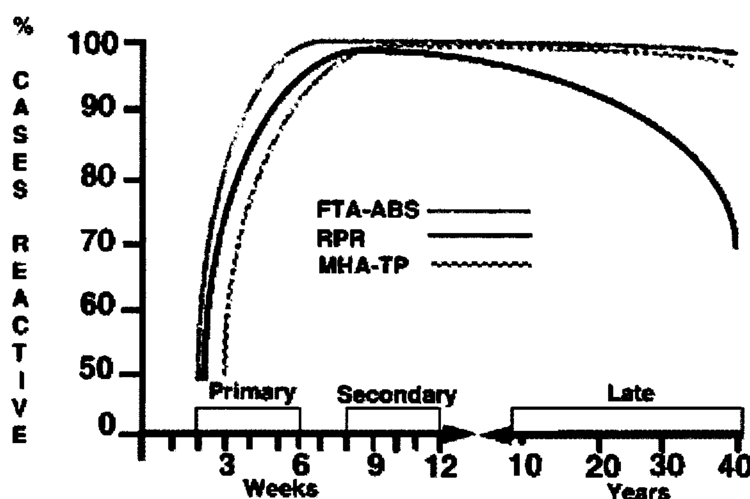



**Fig. 1–7.** Graph depicting that the treponemal tests, FTA-ABS and MHA-TP remain reactive for life and that VDRL test results decrease with time (Larsen *et al.*, 1995), for untreated syphilitic patients. This figure also gives an indication when the various tests become positive.

### *ii.* Treponema pallidum *haemagglutination (TPHA)*

In the TPHA assay, avian erythrocytes coated with *T. pallidum* (Nichols strain) antigen are used (Wicher *et al.*, 1999; Clyne & Jerrard, 2000; Plasmatec, 2003; Giesse

Diagnostics, 2005). Serum or plasma is diluted in diluent containing Reiter treponemes. The sensitised erythrocytes are mixed with diluted plasma or serum in a microtitre plate, and incubated for 45 – 60 minutes (min). Treponemal antibody in the serum sample will cause red blood cells to cross-link (agglutinate) and form a smooth mat at the bottom of the well. If no antibody is present a compact button will be formed due to the settling of non-agglutinating particles. A disadvantage is that the erythrocytes may non-specifically agglutinate with high numbers of heterophile antibodies. TPHA depicts high sensitivity, but TPHA is not sensitive enough for diagnosis of primary syphilis.

Microhaemagglutination assay for *Treponema pallidum* (MHA-TP) works on a similar principle to TPHA, using sheep erythrocytes (Larsen *et al.*, 1995; Wicher *et al.*, 1999). It is being phased out.

### *iii.* Treponema pallidum*-particle agglutination (TP-PA) test*

This test follows the same principle as TPHA, except that it uses purified *T. pallidum* antigen immobilized onto coloured gelatine particles, instead of erythrocytes (Matsumoto *et al.*, 1993; Wicher *et al.*, 1999; Clyne & Jerrard, 2000; Fujirebio Inc., 2006). TP-PA is advantageous to FTA-ABS and MHA-TP, in some aspects. Compared to MHA-TP, TP-PA is easier to performed, has a shorter incubation time, heterophile reactions do not occur, and results are easier to read (Pope *et al.*, 2000). Compared to FTA-ABS, TP-PA does not require a fluorescent microscope and is less subjective (Castro *et al.*, 2001). In at least two studies, TP-PA sensitivity was comparable to that of FTA-ABS sensitivity in primary syphilis (Matsumoto *et al.*, 1993; Castro *et al.*, 2001). However, some literature says that TP-PA is less sensitive than FTA-ABS in untreated primary syphilis, but compares favourably in the other stages (Wicher *et al.*, 1999; Pope *et al.*, 2000; Fujirebio Inc., 2006).

The sensitivities and specificities of some serological tests are summarised in Table 1–2.

**Table 1–2.** Sensitivity and specificity of various non-treponemal and treponemal serological tests, at different stages of untreated syphilis infection (Wicher *et al.*, 1999). These results come from the Centers for Disease Control and Prevention studies.

| Name of test | Sensitivity (%) [e] | | | | Specificity (%) [a, e] | Reference |
|---|---|---|---|---|---|---|
| | Primary | Secondary | Latent | Late | | |
| *Non-treponemal tests* | | | | | | |
| VDRL [b] | 74 – 87 | 100 | 88 – 100 | 37 – 94 | 96 – 99 | Larsen *et al.*, 1995 |
| RPR [b] | 77 – 99 | 100 | 95 – 100 | 73 | 93 – 99 [f] | Larsen *et al.*, 1995 |
| EIA-VDRL [c] | 93 | 100 | 100 | ND* | 97 | Wicher *et al.*, 1999 |
| *Treponemal tests* | | | | | | |
| FTA-ABS [b] | 70 – 100 | 100 | 100 | 96 | 94 – 100 [f] | Larsen *et al.*, 1995 |
| MHA-TP | 69 – 90 | 100 | 97 – 100 | 94 | 97 – 100 | Larsen *et al.*, 1995 |
| TP-PA [d] | 89 | 100 | 100 | ND | 96 | Pope *et al.*, 2000 |

* ND – Not determined

[a] Sera taken from patients with no syphilis.

[b] Tests that are regularly used as diagnostic tools in clinics.

[c] Using the commercial kit, Spirotek Reagin II.

[d] Using the commercial kit, Serodia TP-PA.

[e] The sensitivities and specificities of the different assays listed in this table may not necessarily be comparable to each other as different studies may have used different control populations and 'gold standards' when evaluating the respective tests. For example, if they did not compare the experimental diagnostic test to the 'gold standard' of the rabbit infectivity test (RIT); or to the same combination of tests, as the other studies. See also text below for further explanation.

[f] RPR vs. FTA-ABS specificity: see text below.

It may seem odd at first that the RPR (non-treponemal test) and FTA-ABS (treponemal test) have very similar specificities (Table 1–2), because non-treponemal antibodies or non-treponemal tests are often referred to as non-specific or less-specific for syphilis, and non-treponemal antibodies are produced in response to a wide variety of disorders (section 1.7). On the other hand, treponemal tests are often referred to as being specific.

The similar specificities of RPR and FTA-ABS could perhaps be partially explained by taking these factors into account (Wicher *et al.*, 1999; and personal communication with Dr K Wicher):

- The sera of non-syphilitic patients that were tested to determine specificity of the test were probably taken from a Blood Bank where there were no or very few biological false positive reactions (section 1.7.3) for the non-treponemal tests. This would increase the specificity of the non-treponemal test.

- Specificity depends on the geographical location of the 'healthy' population studied. For this study it was probably the Georgia population, USA. Please see section 1.7.3 for a discussion of specificity and prevalence.
- In the opinion of Wicher *et al.*, 1999, non-treponemal antibodies should be viewed as specific as the treponemal antibodies for syphilis. Treponemal antibodies cannot distinguish between *T. pallidum*, other pathogenic treponemes and many non-pathogenic treponemes, thus rendering the specificities of the RPR and FTA-ABS similar.

### 1.7.3. Screening and confirmatory tests

Normally patients suspected of having syphilis or patients in high-risk areas are screened using non-treponemal serology e.g. VDRL or RPR (Wicher *et al.*, 1999; Clyne & Jerrard, 2000; Goldmeier & Guallar, 2003). This is done since the non-treponemal tests are cheaper and easier to perform, however they are generally considered to be less specific and sensitive than treponemal tests. The preliminary positive diagnosis of syphilis can then be confirmed using the more specific, more expensive and technically difficult treponemal serology. Treponemal tests may also be used when clinically there is a high degree of suspicion that a patient has syphilis even though the screening test result is negative (Lee *et al.*, 2005).

Although the non-treponemal tests are useful as screening tests, they may produce false positive results (Wicher *et al.,* 1999; Clyne & Jerrard, 2000). Reactive non-treponemal tests together with non-reactive treponemal tests are considered biological false positives. These occur because antibodies against phospholipids, including cardiolipin, are produced in a variety of conditions as mentioned in section 1.7.2.

If the prevalence of a disease is low, there are a higher number of false positive results and thus the specificity of the test would be lower (Barker & Rose, 1984; Coggon *et al.*, 1997). Therefore a more specific screening test should be used if the prevalence of disease is known to be low in the area to be screened.

The tests commonly used to diagnose the different stages of syphilis are shown in Table 1–3.

**Table 1–3.** Table showing which tests are appropriate for the different stages of syphilis, according to the different sensitivities and specificities of the diagnostic tests at different stages of syphilis (Table 1–2; Deacon *et al.*, 1966; Escobar *et al.*, 1970; Leclerc *et al.*, 1978; Burke & Schaberg, 1985; Hart, 1986; Larsen *et al.*, 1995; Wicher *et al.*, 1999; Clyne & Jerrard, 2000). Also included are brief reasons as to why these tests are chosen.

| **Stage** | **Test** | **Rationale** |
|---|---|---|
| Primary | -DF or DFA-TP of chancre or lymph node aspirate for early primary syphilis<br>-Serum RPR or VDRL<br>-Confirm positive RPR or VDRL reaction or highly suspected case with FTA-ABS | DF or DFA-TP is used for early primary syphilis, because antibodies are not yet detectable, and approximately 30% of patients with early primary syphilis have negative non-treponemal tests. |
| Secondary and early latent | -Serum VDRL or RPR<br>-Confirm positive reaction or highly suspected case with FTA-ABS or agglutination. | Treponemal and non-treponemal tests generally reach 100% sensitivity when using serum from patients with secondary and early latent syphilis. |
| Late latent | -Serum treponemal test, FTA-ABS or agglutination | Non-treponemal tests reactivity decrease with increasing latency (~ 30% negative VDRL/RPR results occur in untreated late latent/late syphilis) and VDRL antibody titre decreases. The sensitivities of the treponemal tests are nearly 100%. |
| Tertiary | -Serum treponemal test | Approximately 30% of patients will have negative non-treponemal tests. The sensitivities of the treponemal tests are nearly 100%. |
| Neurosyphilis | -Serum treponemal test<br>-CSF protein concentration (suspected if > 40 mg/dl)<br>-CSF mononuclear cell count (suspected if > 5 cells/mm$^3$)<br>-CSF-FTA-ABS<br>-CSF-VDRL | Serum treponemal tests display higher sensitivities than non-treponemal tests during late syphilis. The CSF-FTA-ABS is very sensitive but not specific for neurosyphilis; while the CSF-VDRL is very specific for neurosyphilis, but has a low sensitivity. For further explanation, see section 1.8. |

### 1.7.4. Molecular diagnosis

Since the advent of the polymerase chain reaction (PCR) in the mid-1980s, molecular methods to detect etiological agents have been developed. Molecular methods, for example PCR have been useful to detect the presence of fastidious and slow growing organisms (Louie *et al.*, 2000).

Advantages of PCR include:

- high sensitivity;
- high specificity;
- good reproducibility;
- ability to detect organisms that may not be able to be detected by conventional methods;
- rapidity (able to give same-day results). Rapidity has implications for infection control and earlier administration of therapy;
- ability to detect more than one organism in one reaction, by multiplex PCR assays.

Limitations or disadvantages of PCR include:

- false positive results e.g. due to amplification of contaminating DNA;
- false negative results e.g. due to inhibitors in some clinical samples;
- the high expense e.g. expensive equipment and reagents, the need for pre-PCR and post-PCR separate rooms;
- positive PCR results not yet validated for some infectious diseases e.g. the result could be positive because of active or latent disease or the presence of non-viable organisms. With this in mind, PCR should not be used to monitor therapy.

Examples of organisms for which PCR diagnostic tests have been developed and are commercially available, are for the bacteria, *Chlamydia trachomatis*, *Mycobacterium tuberculosis*, *Neisseria gonorrhoeae*; the viruses, cytomegalovirus, enterovirus, herpes simplex virus and human immunodeficiency virus (HIV); and the parasite *Toxoplasma gondii* (Louie *et al.*, 2000).

While PCR is probably the most well recognised of the nucleic acid amplification methods, a variety of other nucleic acid tests have also been described. These include branched DNA (bDNA), ligase chain reaction (LCR), nucleic acid sequence-based amplification (NASBA), reverse transcription-polymerase chain reaction (RT-PCR),

strand-displacement amplification (SDA), and transcription-mediated amplification (TMA) (Versalovic & Lupski, 2002).

Given that *T. pallidum* cannot be cultured *in vitro* it is not surprising that a number of investigators have described molecular assays for the detection of the organism.

#### *1.7.4.1. Polymerase chain reaction (PCR)*

There have been several reports of investigators using PCR to detect *T. pallidum* DNA (Hay *et al.*, 1990; Burstain *et al.*, 1991; Grimprel *et al.*, 1991; Noordhoek *et al.*, 1991; Orle *et al.*, 1996; Liu *et al.*, 2001). The *T. pallidum* genes that have been amplified include the: 4D gene (Hay *et al.*, 1990); *tmpA* gene (Hay *et al.*, 1990); *bmp* gene (Noordhoek *et al.*, 1991); and *tpp47* gene (Burstain *et al.*, 1991; Grimprel *et al.*, 1991; Orle *et al.*, 1996).

Under certain conditions PCR was more sensitive and specific than other techniques. These results are summarised in Table 1–4. It is difficult to compare the methods since the techniques are not standardised, and may differ in, for example, DNA extraction methods and primers used. As yet, PCR to detect *T. pallidum* is not used routinely in clinics due to the lack of a well-validated assay (Larsen *et al.*, 1995). It is interesting to note that of all of the advantages of PCR, the RIT is more sensitive than the PCR technique (Centurion-Lara *et al.*, 1997).

#### *1.7.4.2. Reverse transcription-polymerase chain reaction (RT-PCR)*

There is presently one report of an RT-PCR method for detecting *T. pallidum* (Centurion-Lara *et al.*, 1997). Human CSF was spiked with *T. pallidum* and total ribonucleic acid (RNA) was extracted. A 366 bp region within the 16S ribosomal RNA (rRNA) was targeted in the reverse transcription (RT)-step, and the resulting cDNA was amplified during PCR. The amplicons obtained indicated the presence of *T. pallidum*. This method was more sensitive than PCR targeting the *tpp47* gene. It was able to detect a single treponeme, reproducibly (Table 1–4). The RT-PCR procedure must still be evaluated using clinical samples.

**Table 1–4.** Table comparing the sensitivities and specificities and various other parameters of PCR and RT-PCR assays performed on *T. pallidum* samples.

| Reference | Sample type | DNA / RNA extraction method | Detection method | Sensitivity [a] | Specificity [b, c] | Primers used |
|---|---|---|---|---|---|---|
| **PCR** | | | | | | |
| ***tmpA* gene** – coding for the 45 kd surface protein | | | | | | |
| Hay *et al.*, 1990 | Rabbit-derived *T. pallidum* suspended in human CSF | Boiling with lysis buffer | DNA-DNA hybridisation (Southern blot) | 65 treponemes/500 µl CSF | 13 organisms:<br>-*T. pertenue* : +<br>-4 other treponemes and 8 non-treponemes: – | PHT1 & PHT2 |
| **4D gene** | | | | | | |
| Hay *et al.*, 1990 | Rabbit-derived *T. pallidum* suspended in human CSF | Boiling with lysis buffer | Southern blot | 65 treponemes/500 µl CSF | Same as for the *tmpA* gene. | PHD1 & PHD2 |
| ***bmp* gene** – coding for the 39 kd basic membrane protein | | | | | | |
| Noordhoek *et al.*, 1991 | Initial investigations – rabbit-derived *T. pallidum* suspended in phosphate-buffered saline | SDS, proteinase K, phenol, ethanol precipitation | Southern blot | 1 fg DNA or<br>1 *T. pallidum* organism | | PCR 1: TP 7 & TP 8 |
| | Lysed rabbit-derived *T. pallidum* in CSF | SNF: adsorption to diatoms in the presence of guanidine thiocyanate<br>Pellet: lysis buffer | Agarose-EtBr* gel | SNF: 100 treponemes/ml CSF<br>Pellet: $10^4 – 10^5$ cells/ml CSF | | Nested PCR:<br>PCR 1: TP 7 & TP 8<br>PCR 2: TP 3 & TP 4 |
| | *T. pallidum* in CSF (clinical samples) from patients with neurosyphilis. | Freeze-thawed CSF.<br>SNF: as above<br>Pellet: as above | Southern blot | 7 out of 27 cases where *T. pallidum* was detected, before treatment | 30 organisms:<br>-*T. pallidum* subsp. *pertenue* CDC 2575 and 352 Pariaman; and *T. pallidum* SS3: +<br>-4 other treponemes and 23 non-treponemes: – | Nested PCR:<br>PCR 1 & PCR 2 |

**Table 1–4 continued.**

| Reference | Sample type | DNA / RNA extraction method | Detection method | Sensitivity [a] | Specificity [b, c] | Primers used |
|---|---|---|---|---|---|---|
| ***tpp47* gene**, encoding the 47 kd membrane immunogen | | | | | | |
| Burstain *et al.*, 1991 | Initial investigations – rabbit-derived *T. pallidum* suspended in saline and rabbit serum | Two different methods used:<br>-boiling<br>-boiling with alkaline lysis buffer and phenol-chloroform extraction. | DNA-DNA hybridisation (dot blot) | 0.01 pg – 0.1 pg or 1 – 10 *T. pallidum* organisms/50 µl sample. | 25 organisms:<br>-*T. pallidum* subsp. *pertenue*: +<br>-Other 2 treponemes and 22 non-treponemes: – | 47-1 & 47-2 |
| | -Serum<br>-CSF<br>-Amniotic fluid<br>from patients with syphilis | Boiling with alkaline lysis buffer and phenol-chloroform extraction. | Dot blot | 10 out of 12 positive by PCR. Two of these were confirmed by RIT. (Other data not given.) | | 47-1 & 47-2 |
| Grimprel *et al.*, 1991 | -Amniotic fluid of pregnant syphilitic women<br>-Sera and CSF of suspected congenital syphilitic neonates | Four different methods used on selected samples: boiling; low-spin separation; alkaline lysis; spin extraction. | Dot blot | Percentages of PCR positive relative to RIT positive: 100% (amniotic fluid); 71% (CSF); 67% (sera); 80% (average). | 100% specific for *T. pallidum* compared to RIT (i.e. no false positives in PCR). | 47-1 & 47-2 |
| ***pol A* gene** – coding for DNA polymerase I | | | | | | |
| Liu *et al.*, 2001 | Initial investigations – rabbit-derived *T. pallidum* in water | Blood-Tissue Kit or DNA Mini Kit (Qiagen) | Agarose-EtBr gel | 10 – 25 organisms | 59 organisms:<br>-*T. pallidum* subsp. *pertenue* and *T. pallidum* subsp. *endemicum* : +<br>-3 other treponemes and 54 non-treponemes: – | Two different PCR reactions:<br>F1 & R1; F2 & R2 |
| | | | ABI 310 Prism Genetic Analyzer to detect fluorescent-labelled amplicons | 1 *T. pallidum* organism | | Two different PCR reactions: F1 & R1; F2 & R2 |
| | Genital ulcers (112) | Blood-Tissue Kit or DNA Mini Kit (Qiagen) | Agarose-EtBr gel | 95.8% compared to multiplex PCR [d] (Roche) | 95.7% compared to multiplex PCR [d] (Roche). | Two different PCR reactions: F1 & R1; F2 & R2 |

**Table 1–4 continued.**

| Reference | Sample type | DNA / RNA extraction method | Detection method | Sensitivity [a] | Specificity [b, c] | Primers used |
|---|---|---|---|---|---|---|
| **RT-PCR** | | | | | | |
| **16S rRNA** | | | | | | |
| Centurion-Lara *et al.*, 1997 | Initial investigations – rabbit-derived *T. pallidum* in water. Organisms were diluted before RNA extraction. | RNAzol B method | Southern blot | 1 *T. pallidum* organism | 13 organisms: -*T. pallidum* subsp. *pertenue*: + -2 other treponemes and 10 non-treponemes: – | Sense & antisense primers |
| | Initial investigations – rabbit-derived *T. pallidum* in water. RNA was extracted then RNA diluted. | | | $10^{-2}$ to $10^{-3}$ *T. pallidum* equivalents | | Sense & antisense primers |
| | Human CSF spiked with rabbit-derived *T. pallidum*. RNA was extracted then RNA diluted. | | | $10^{-2}$ *T. pallidum* equivalents | | Sense & antisense primers |

* EtBr – ethidium bromide; SNF – supernatant fluid.

+ = positive result; – = negative result.

[a] The sensitivity percentages in this table cannot necessarily be directly compared to each other as there are variables. For example, different reference tests were used for the different studies, e.g. the authors did not compare PCRs to the same 'gold test' of RIT.

[b] For the same reason the specificity percentages in this table cannot necessarily be directly compared to each other. For example, the authors did not all use the same set of organisms and numbers of organisms tested, varied.

[c] For specificity testing, DNA was extracted as per standard methods (Maniatis *et al.*, 1982 or Sambrook *et al.*, 1989), or using the same method as for the clinical sample.

[d] Multiplex PCR for *T. pallidum*, herpes simplex virus types 1 and 2, and *Haemophilus influenzae* (Roche).

## 1.8. DIAGNOSIS OF NEUROSYPHILIS

Neurosyphilis is difficult to diagnose, as it may be asymptomatic. It is only symptomatic in 4 – 9% of patients with untreated syphilis (Hook & Marra, 1992). When symptomatic, it manifests non-specific symptoms except for the Argyll Robertson pupil and tabes dorsalis (Tramont, 2000). Another difficulty is that it can be confused with a variety of other neurological conditions or CNS infections (Centurion-Lara *et al.*, 1997). For example, neurosyphilis and tuberculous meningitis share symptoms, although requiring very different treatment strategies. A list of common differential diagnoses is shown in Table 1–5.

**Table 1–5.** Differential diagnoses of neurosyphilis (Tramont, 2000).

| Differential diagnoses |
|---|
| Degenerative neurological processes |
| Fungal meningitis |
| Parasitic meningitis |
| Sarcoid meningitis |
| Tumours |
| Subdural haematoma |
| Alzheimers disease |
| Multiple sclerosis |
| Chronic alcoholism |
| Any disorder affecting the vasculature of the central nervous system |

Diagnosis of neurosyphilis in HIV-positive patients can be even more complex. Several studies suggest that, with HIV co-infection: neurosyphilis occurs more frequently, progresses more rapidly and manifests unusual symptoms (e.g. Johns *et al.*, 1987, Katz & Berger, 1989, Musher *et al.*, 1990; Tomberlin *et al.*, 1994). Additionally some HIV-positive patients give a negative serological test when tested for syphilis (e.g. Hicks *et al.*, 1987; Blum *et al.*, 2005; Kingston *et al.*, 2005). Diagnosis of neurosyphilis in HIV-positive patients is additionally confounded by the fact that some may have CSF abnormalities even without syphilis (Singh & Romanowski, 1999).

Currently, a diagnosis of neurosyphilis requires (Larsen *et al.*, 1995; Wicher *et al.*, 1999; Clyne & Jerrard, 2000):

- positive serum treponemal serology;
- CSF cell count of more than 5 mononuclear cells/mm$^3$ (i.e. lymphocytic pleocytosis (increase) in the CSF);
- raised CSF protein levels of more than 40 mg/dl;
- positive CSF-FTA-ABS; and
- positive CSF-VDRL.

However, serological diagnosis of neurosyphilis is seldom as straightforward.

The CSF-VDRL test is highly specific but unfortunately has a low sensitivity of 22 – 70% (Burke & Schaberg, 1985; Hart, 1986). With regards to specificity, a false positive CSF-VDRL would only really occur if CSF is contaminated with blood (Davis & Sperry, 1979; Burke & Schaberg, 1985). CSF-VDRL compared to CSF-RPR, is preferred because RPR yields a high proportion of false positive results (Margo & Hamed, 1992).

The treponemal test, CSF-FTA-ABS is very sensitive (Deacon *et al.*, 1966; Escobar *et al.*, 1970; Leclerc *et al.*, 1978), but not specific for neurosyphilis (Jaffe *et al.*, 1978). It is not specific because treponemal antibodies from serum may diffuse into CSF, thus resulting in a positive CSF-FTA-ABS even if there is no active neurosyphilis, or because of antibodies remaining in CSF after patients have been adequately treated for early or latent syphilis (Jaffe *et al.*, 1978; LaFond & Lukehart, 2006). Some authors suggest that CSF-FTA-ABS is limited to excluding neurosyphilis when a negative test result is obtained (LaFond & Lukehart, 2006).

Thus while a reactive CSF-VDRL is diagnostic of neurosyphilis, a negative CSF-VDRL does not rule out neurosyphilis (Jaffe *et al.*, 1978; Larsen *et al.*, 1995). A negative CSF-FTA-ABS test generally excludes neurosyphilis (Jaffe *et al.*, 1978).

The main problem, however, is that a positive CSF-FTA-ABS test does not confirm the diagnosis of active neurosyphilis. Thus the result of a negative CSF-VDRL and a positive CSF-FTA-ABS may cause clinical confusion. These patients are in all likelihood usually treated for neurosyphilis since there is currently no other clinically practical test to confirm the diagnosis. The 'gold standard', RIT, could confirm it, but this procedure is expensive, takes 3 – 6 months to diagnose the disease, and is used only in certain research laboratories.

Taking these various factors into account, it is apparent that there is a need for a rapid, sensitive and specific diagnostic test for neurosyphilis. This project will aim to develop a diagnostic assay using a molecular method, RT-PCR.

## 1.9. MOTIVATION FOR USING RT-PCR

The RT-PCR technique (section 1.7.4.2) has several advantages in diagnosing syphilis, especially in the CSF, compared to PCR or RIT.

The RT-PCR method is more sensitive than PCR. RT-PCR was able to detect $10^{-2}$ *T. pallidum* equivalents in CSF while the minimum number of organisms required for detection by PCR is 1 – 10. This RT-PCR process is possibly more sensitive as most bacterial species have a high copy number of 16S rRNA per cell. It is also a very specific test (Centurion-Lara *et al.*, 1997).

Some researchers have noticed that the sensitivity of *T. pallidum* detection is lower when they are suspended in CSF, compared to, for example, a saline buffer (Hay *et al.*, 1990; Burstain *et al.*, 1991; Noordhoek *et al.*, 1991). This could be because when bacteria are concentrated by centrifugation, some inhibitors of PCR present in CSF are also brought down into the pellet (Noordhoek *et al.*, 1991). This RT-PCR technique would be advantageous because it is very likely that it would still be sensitive enough to detect *T. pallidum*, even if some inhibitors are present.

Additionally, when performing the RT-PCR assay, after lyses of organisms during RNA extraction, the sample can be diluted 100-fold more than for PCR (Centurion-Lara *et al.*, 1997). This would then further dilute out any inhibitors that may be present in CSF samples.

RT-PCR would be a rapid test compared to RIT. RIT takes weeks to months to diagnose syphilis (section 1.7.1.3), whereas the RT-PCR procedure would take one to two days.

RT-PCR (compared to PCR) can be advantageous because it could distinguish live from dead organisms, as RNA is rapidly degraded after cells die. In an experiment, it was shown that *T. pallidum* DNA persisted for up to 100 d after infection in rabbits with live treponemes; and for 16 d when using heat-killed treponemes (Centurion-Lara *et al.*, 1997).

Additionally, several investigations have indicated that, with HIV co-infection: neurosyphilis occurs more frequently, progresses more quickly, unusual symptoms occur, and some serological results for syphilis are negative (section 1.8). Therefore RT-PCR diagnostic tests may be useful for HIV-positive patients.

However, to date, RT-PCR has only been tested on human CSF spiked with *T. pallidum* and not tested on CSF samples from patients suspected to have neurosyphilis (Centurion-Lara *et al.*, 1997). Therefore, there is a need to validate the

RT-PCR assay when using clinical samples, and ideally having a test in which RNA extraction and RT-PCR are monitored.

## 1.10. SUMMARY

The bacterium *Treponema pallidum* causes the disease syphilis, which can be transferred sexually, or from mother to foetus. Although there are several diagnostic tests to determine whether a patient has syphilis, there is not a sensitive and specific diagnostic test for neurosyphilis.

Neurosyphilis can mimic other diseases, or give non-specific symptoms, or be asymptomatic. Also, diagnosis in HIV-positive patients can be additionally more difficult. The highly specific CSF-VDRL is not very sensitive. On the other hand, the highly sensitive CSF-FTA-ABS is not specific. Following on from this, the main problem is that a positive CSF-FTA-ABS test does not confirm the diagnosis of active neurosyphilis, therefore a negative CSF-VDRL and positive CSF-FTA-ABS can result in diagnostic confusion. The patient will probably be treated for neurosyphilis because there is no other test to determine the correct diagnosis. This therefore makes the need for a sensitive, specific and rapid diagnostic assay more important.

RT-PCR amplification of an internal region of *T. pallidum* 16S rRNA was shown to be very sensitive ($10^{-2}$ *T. pallidum* equivalent of RNA) for the detection of *T. pallidum* suspended in CSF. However, there is a need to validate the RT-PCR assay when using clinical samples, and ideally having a test in which RNA extraction and RT-PCR are monitored.

## 1.11. PROJECT SUMMARY AND DIAGNOSTIC SYPHILIS ASSAY PRINCIPLE

The aims of this project were to develop, optimise and validate an assay for the detection of *T. pallidum* in CSF samples.

In order to accomplish the above, the following had to be performed:

- Construct the positive control plasmid (Chapter 2).
- Develop and optimise the assay, which involved (Chapter 3)
  - Determination of the amount of *Escherichia coli* positive control cells used to spike clinical samples;
  - Determination of the sensitivity of the assay;
  - Optimisation of the RNA extraction, and RT-PCR steps;
  - Determination of the specificity of the assay.
- Validate the assay using clinical CSF samples (Chapter 4).

This project was approved by the UCT Research Ethics Committee (REC REF 338/2005).

The diagnostic assay was envisaged as follows: An *E. coli* positive control would be added to the clinical samples, to monitor the RNA extraction and RT-PCR steps. The positive control plasmid within this *E. coli* contains *T. pallidum* primer binding sites but yields a larger product than if amplification is performed on *T. pallidum* nucleic acid. This enables the positive control to be distinguished from *T. pallidum*. Total RNA would be extracted and amplified using RT-PCR, and the products electrophoresed on an agarose-ethidium bromide gel, to determine whether the sample contained *T. pallidum*. For an overview of the assay, see Fig. 1–8.

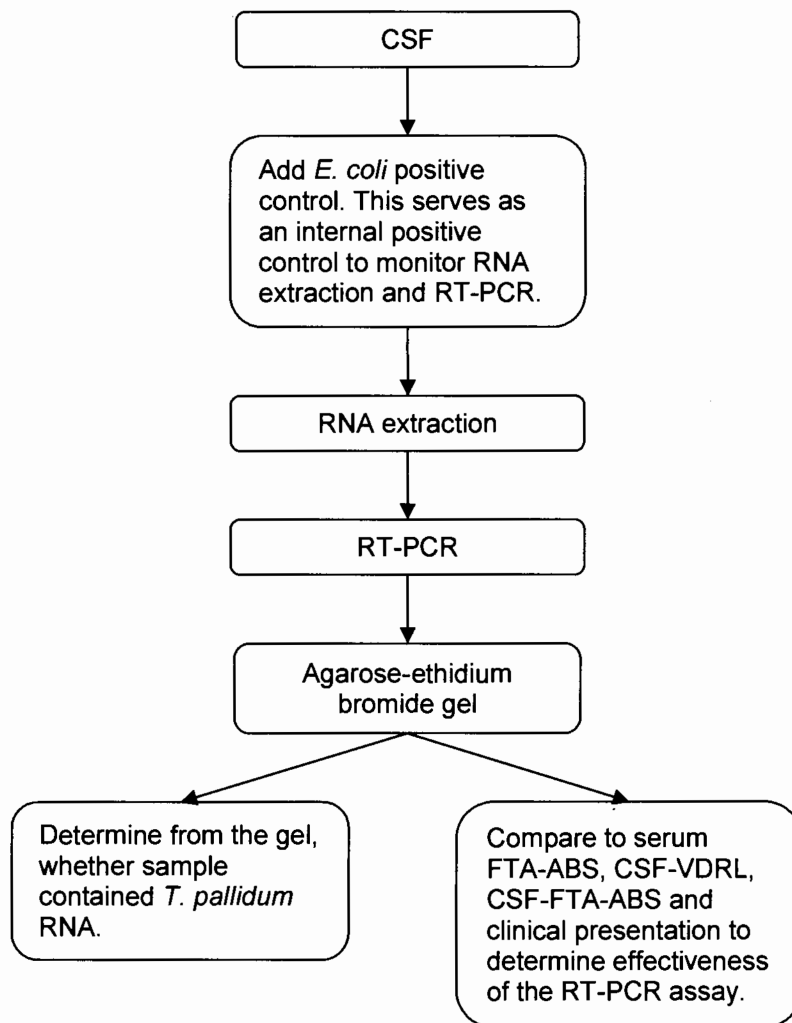



**Fig. 1–8.** Diagrammatic representation of the principle of the diagnostic assay. *E. coli* positive control contains the positive control plasmid.

If the assay proves to be sensitive and specific, it is hoped that it can be used as a routine diagnostic test.

# 2. CHAPTER TWO – CONSTRUCTION OF A POSITIVE CONTROL PLASMID

## 2.1. INTRODUCTION

An important feature of a reliable diagnostic assay is the inclusion of a positive control, to indicate that the assay is working. In the proposed *T. pallidum* RT-PCR diagnostic assay (Fig. 1–8 and section 1.11), *E. coli* harbouring a positive control plasmid would be added to the samples prior to processing, following which, RNA extraction and RT-PCR would be performed. This positive control plasmid is to be constructed such that amplicons generated using *T. pallidum* specific primers in the RT-PCR assay, will be considerably larger than the amplicons generated from *T. pallidum*. This *E. coli* positive control would be added to the clinical samples to monitor both the RNA extraction and RT-PCR steps.

Spiking each clinical sample with the *E. coli* positive control before performing RNA extractions and RT-PCR would be an advantage as it would act as a control for each RT-PCR reaction. The decision to employ *E. coli* positive control as an internal control for the RT-PCR assays, was made because:

- addition of *T. pallidum* RNA in a separate RT-PCR control tube would not monitor RNA extraction and RT-PCR of the clinical sample;
- addition of *T. pallidum* DNA in a separate RT-PCR control tube would not control for RNA extraction nor the reverse transcription process;
- use of viable *T. pallidum* cells would not be practical as *T. pallidum* cannot be cultured on media and it can only be cultured *in vivo*, most commonly in rabbit testes.

This chapter describes the construction of a positive control plasmid. A diagrammatic representation of the proposed positive control plasmid is shown in Fig. 2–1. In summary, it was constructed in two stages; as follows (Fig. 2–2):

Phase I plasmid: In this stage the *T. pallidum* primer binding sites were inserted in a vector. An internal region (366 bp) of the *T. pallidum* 16S rRNA gene was amplified by PCR from *T. pallidum* genomic DNA using the sense and antisense primers (Centurion-Lara *et al.*, 1997). The 366 bp DNA fragment was ligated with a vector. Since the PCR product was cloned directly into the vector, the recombinant plasmid would contain the *T. pallidum* primer binding sites.

Phase II plasmid (positive control plasmid): In order to obtain a positive control plasmid amplicon of 2 – 3 times the size of the *T. pallidum* amplicon (using the sense and antisense primers), spacer DNA was inserted into the phase I plasmid to differentiate between amplification products originating from *T. pallidum* and the positive control. The phase I plasmid was linearised within the cloned 366 bp 16S rDNA insert. Spacer DNA of 400 – 800 bp was ligated with the linearised phase I plasmid. The phase II plasmid therefore still contains the *T. pallidum* primer binding sites, but its PCR product is of a greater size than 366 bp. An *E. coli* clone harbouring this phase II plasmid will be used to spike clinical samples.

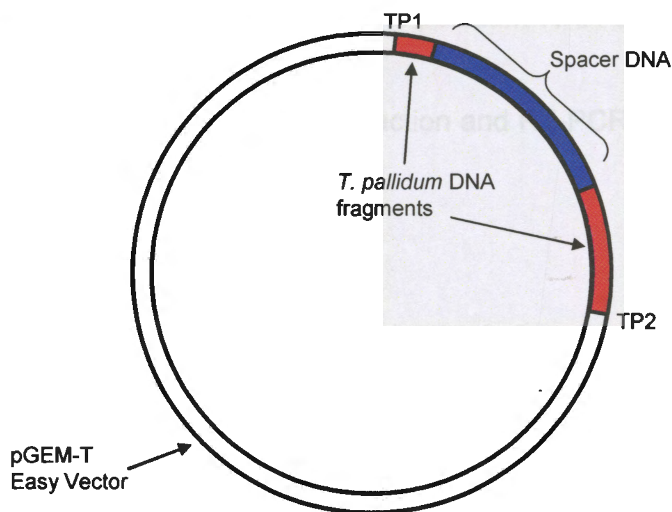
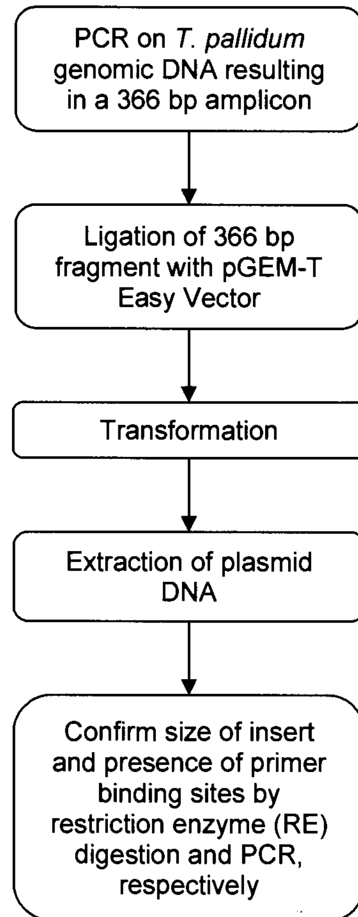



**Fig. 2–1.** Diagram showing the proposed positive control plasmid components. TP1 and TP2 represent the *T. pallidum* primer binding sites. Spacer DNA would allow for differentiation between nucleic acids originating from *T. pallidum* and the positive control on the basis of size.

The size of the 16S rRNA target region of *T. pallidum* is 366 bp (Centurion-Lara *et al.*, 1997). However, the amplicon size generated using RNA transcribed from the positive control plasmid would be 2 – 3 times the size of the *T. pallidum* amplicon, i.e. approximately 1000 bp. After performing the assay and electrophoresing the amplicons on an agarose-EtBr gel, the following results and interpretations would be possible:

- no bands – RNA extraction and/or RT-PCR did not work, and any results obtained are invalid.
- 1000 bp band only – RNA extraction and RT-PCR were successful but the sample did not contain *T. pallidum*, or *T. pallidum* RNA concentration was too low to be detected by the assay.
- 1000 bp and 366 bp bands – RNA extraction and RT-PCR were successful and the sample did contain *T. pallidum*.



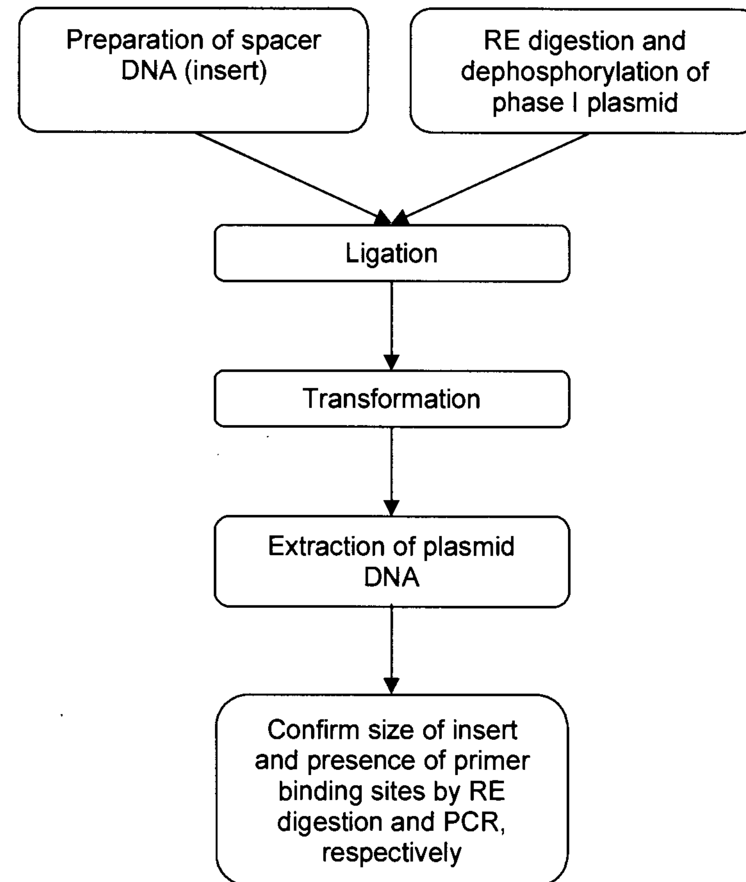




**Fig. 2–2.** Graphical representation summarising the two major phases to produce the positive control plasmid.

## 2.2. MATERIALS AND METHODS FOR CONSTRUCTION OF PHASE I PLASMID

### 2.2.1. Bacterial strains, plasmids and solutions

Bacterial strains and plasmids used in construction of a phase I plasmid, as well as their important characteristics and uses, are listed in Table 2–1. Solutions and media referred to in this thesis can be found in Appendix A.

**Table 2–1.** Bacterial strains and plasmids used in construction of a phase I plasmid.

| Bacterial strains / plasmids | Relevant Feature | Reference |
|---|---|---|
| **Bacterial strains** | | |
| *E. coli* JM109 | This strain, when harbouring the appropriate plasmid and inoculated on the appropriate media, can be used to distinguish non-transformants, recombinants and non-recombinants from each other, during the cloning process. *E. coli* JM109 genomic DNA codes for an incomplete and therefore inactive α-peptide of β-galactosidase; and a complete ω-peptide of the β-galactosidase enzyme – a characteristic which is utilised in the cloning process. For more detail see section 2.2.6. | Ullmann *et al.*, 1967; Ausubel *et al.*, 1987. |
| **Plasmids** | | |
| pGEM-T Easy Vector | The pGEM-T Easy Vector (Appendix D) is linear, and has 3'-T terminal overhangs preventing recircularisation of this vector, and providing compatible overhangs with PCR products if *Taq* polymerase added a 5'-A overhang. The vector is prepared by digesting Promega's pGEM-T Easy Vector with *Eco*RV (within the multiple cloning site, MCS) and adding a 3'-T to both ends.<br>The MCS is within the coding sequence for the α-peptide of β-galactosidase. The vector contains the ampicillin resistance gene coding for β-lactamase.<br>Inserts can be sequenced using the following primers: SP6 promoter primer, T7 promoter primer, pUC/M13 forward primer or pUC/M13 reverse primer. | Promega, 2003 |
| pKS | A 2 959 bp vector derived from pUC19. It contains a portion of the *lacZ* gene (coding for the α-peptide of β-galactosidase) interrupted by a MCS; and an ampicillin resistance gene. | Coyne *et al.*, 2004 |

### 2.2.2. Amplification of a 366 bp region from *T. pallidum* genomic DNA by PCR

In 1984 Kary Mullis invented PCR, which is a technique used to amplify DNA sequences (Stryer, 1995; Grunenwald, 2003). PCR consists of three steps (Fig. 2–3), namely heat denaturation of template (94 or 95°C for 30 seconds (s) – 1 min); binding of primers to template (the temperature is determined according to the primers' melting temperatures); and extension of primers (72°C). These three steps are repeated 20 – 40 times. The primers are generally complementary to the region flanking the target sequence (i.e. the reverse primer is complementary to a 3' section of the sense target template, and the forward primer is complementary to a 3' portion of the antisense target sequence). Thus, the sequence flanking the target DNA generally needs to be known, for construction of the primers. Before the first cycle, an initial step of 95 or 94°C for 2 min or more is performed, to denature the template. One final extension period of about 5 min at 72°C is performed in case extension has not been completed. The PCR reaction is performed using a thermal cycler. Components for PCR include: deoxynucleoside triphosphates (dNTPs), forward and reverse primers, thermostable DNA polymerase, magnesium chloride ($MgCl_2$), buffer and template. During the first 20 – 40 cycles, exponential amplification of the region of interest occurs. For example, after 20 cycles, the target molecule is amplified by $2^{20}$ i.e. approximately a million-fold.

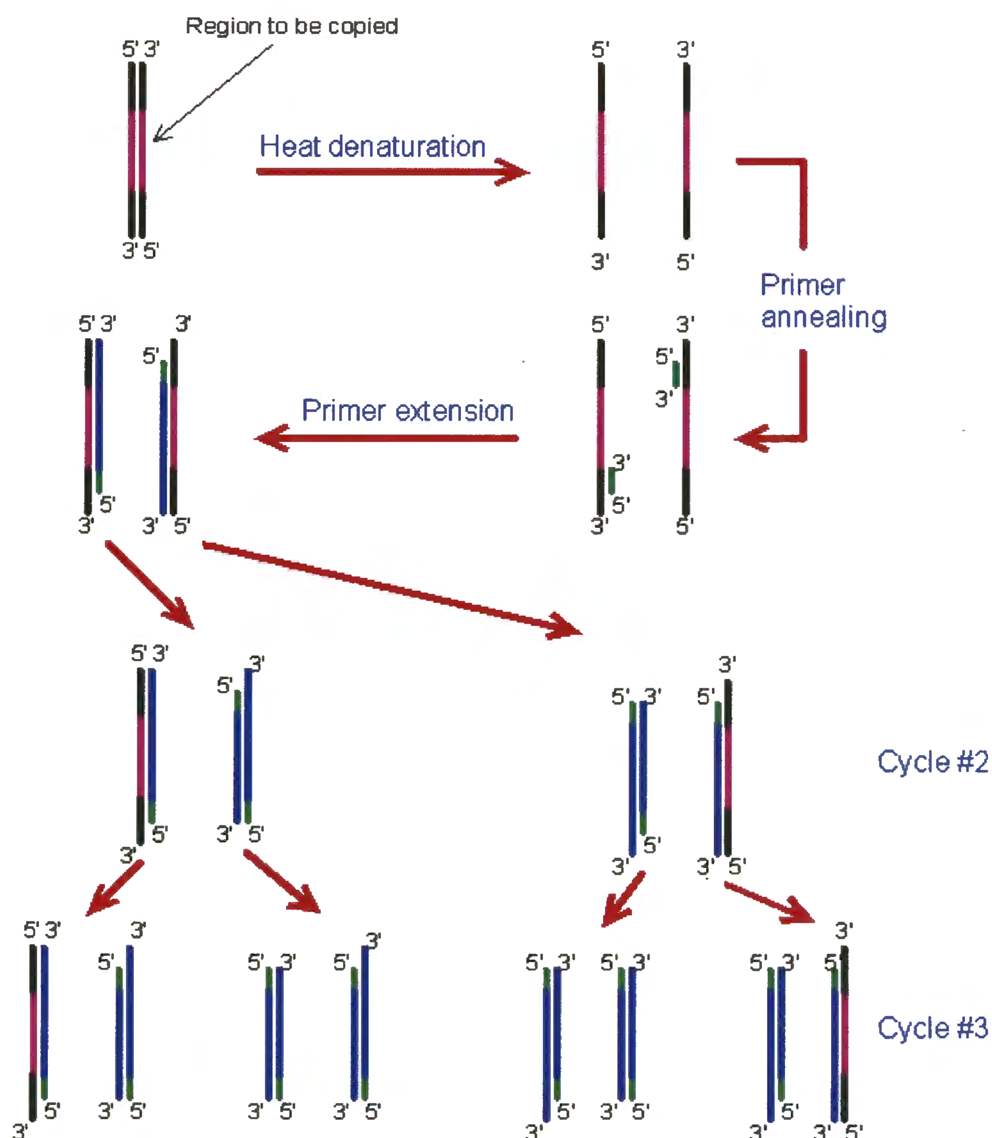



**Fig. 2–3.** Diagram summarising three steps of the polymerase chain reaction and showing how exponential amplification of the target DNA occurs (http://www.web-books.com/MoBio/Free/Ch9E.htm, accessed on 22 May 2007).

The above-mentioned PCR technology was used to amplify a 366 bp internal region (position 203 – 568) of the 16S rRNA gene of *T. pallidum*. The PCR reaction was performed in a total volume of 50 µl, with final concentrations of : 1X Reaction Buffer (JMR Holdings), 1.5 mM $MgCl_2$ (JMR Holdings), 1.5 U Supertherm *Taq* DNA Polymerase (JMR Holdings) per reaction, 0.4 mM each dNTP (ABgene) and 0.5 pmol/µl each of sense and antisense primers (Department of Molecular and Cell Biology, University of Cape Town (UCT)).

The 'sense primer' is *T. pallidum* specific corresponding to positions 203 – 226 of the *T. pallidum* 16S rRNA gene (5'-CTC TTT TGG ACG TAG GTC TTT GAG-3') (Centurion-Lara *et al.*, 1997). The 'antisense primer' is *Treponema* group specific,

corresponding to positions 549 – 568 (5'-TTA CGT GTT ACC GCG GCT GG-3'). The nucleosides A, C, G and T depict adenosine, cytidine, guanosine and thymidine, respectively. The primers will be referred to as sense and antisense primers in this thesis.

Template used was either $10^3$, $10^5$, $10^7$ or $10^9$ *T. pallidum* (Nichols strain) genomic DNA equivalents (kindly donated by Prof. S. Lukehart, University of Washington, USA) or water as a negative control. The negative control was used to determine whether any of the PCR reagents were contaminated; and was performed for all PCR reaction sets described in this thesis.

The PCR cycling conditions employed were as follows: an initial denaturation step at 94°C (2 min); 40 cycles of denaturation at 94°C (1 min), annealing of primers to template at 62°C (1 min) and extension at 72°C (1.5 min); and a final extension step at 72°C (5 min) using a Thermal Cycler 2720 (Applied Biosystems). The PCR cycles and reagent concentrations that were used, were modified from Centurion-Lara *et al.* (1997).

The melting temperature ($T_m$) of the antisense primer is 64°C and that of the sense primer is 70°C, calculated by using the formula $T_m$= 4(G & C) & 2(A & T), and in agreement with the program, DNAMAN (Lynnon BioSoft, Version 4.0). Generally, annealing temperature ($T_a$) is 5°C below the lower $T_m$ of the two primers (Coyne *et al.*, 2004). In this case the $T_a$ would be 59°C. Possibly Centurion-Lara *et al.* (1997) used another formula to calculate $T_m$ or $T_a$. However, it was elected to use 62°C as this $T_a$ had previously been described and used successfully (Centurion-Lara *et al.*, 1997).

### 2.2.3. Agarose gel electrophoresis

The PCR products were electrophoresed on a 2% (w/v) agarose gel containing 0.5 µg of ethidium bromide (EtBr) (Boehringer Mannheim) per ml of 1X Tris-acetate-ethylenediaminetetraacetic acid (TAE) buffer (Appendix B.1). Ethidium bromide, containing a planar group, intercalates between DNA base pairs, and when subject to ultraviolet (UV) irradiation, the DNA-EtBr complex emits fluorescent light. The DNA-EtBr complex produces much more fluorescence than unattached EtBr (Sambrook *et al.*, 1989). Thus, DNA was visualised by subjecting the gel to UV irradiation.

All agarose gels mentioned in this thesis contained 2% (w/v) of agarose, unless otherwise stated.

### 2.2.4. Purification of DNA

Electrophoresed PCR products were visualised using UV (avoiding prolonged exposure), excised from the gel with a sterile scalpel blade, and gel-purified according to the QIAquick Gel Extraction Kit protocol (Qiagen, 2002). The silica-gel membrane in the column selectively binds DNA, when the solution is at a high salt concentration and a low pH. Salt concentration is adjusted by the buffers, and, if necessary, pH can be adjusted by adding sodium acetate. The buffers and centrifugation aid to remove contaminants. The DNA was eluted in 10 mM Tris-Cl, pH 8.5 (Qiagen). The purified DNA was quantified using lambda (λ) genomic DNA standards (Appendix B.2).

### 2.2.5. Ligation of the 366 bp fragment with the pGEM-T Easy Vector

The ligation reaction, in which the pGEM-T Easy Vectors and 366 bp fragments were ligated, was performed in a volume of 10 µl, containing : 1X Rapid Ligation Buffer (Promega), 50 ng pGEM-T Easy Vector (Promega), 3 Weiss units T4 DNA Ligase (Promega) and 25 ng 366 bp PCR product. A positive ligation control with insert DNA in the *lacZ* gene, to check efficiency of ligation, was performed according to manufacturer's instructions (Promega, 2003). Ligation reactions were incubated for 4 d at 4°C.

### 2.2.6. Transformation

*E. coli* JM109 competent cells were prepared by calcium chloride shock treatment (Appendix B.3). Subsequently, *E. coli* JM109 competent cells were transformed with DNA (Appendix B.4). To four separate tubes one of the following was added:

- All of the experimental ligation mix;
- All of the positive ligation control;
- 0.1 ng circular pKS vector;
- no DNA.

The appropriate amount of cells was inoculated on media (Table 2–2). Cells transformed with experimental ligation mix, positive ligation control or circular pKS vector were inoculated on 2X yeast-tryptone (2X YT) agar, supplemented with ampicillin (Ranbaxy), isopropyl-β-D-thiogalactopyranoside (IPTG) (Bioline) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (Roth) all at a final

concentration of 100 μg/ml each. Cells transformed with no DNA were inoculated on 2X YT agar containing 100 μg/ml ampicillin, or on 2X YT agar without ampicillin. Agar plates were incubated at 37°C overnight.

Ampicillin, IPTG and X-gal were added to distinguish non-transformants, recombinants (transformants) and non-recombinants (transformants) from each other. Ampicillin distinguishes transformants from non-transformants (Ullmann *et al.*, 1967; Ausubel *et al.*, 1987). When a colony has grown on media containing ampicillin, it indicates that the cells in the colony contain an ampicillin-resistance gene (in this case, the β-lactamase gene on the plasmid, pGEM-T Easy Vector or phase I plasmid), and therefore that it is a transformant. Non-transformants i.e. cells without plasmid, will not be able to grow on ampicillin-containing media.

X-gal and IPTG are used to distinguish recombinants (white colonies) from non-recombinants (blue colonies). In a non-recombinant, the β-galactosidase gene (*lacZ*) on the plasmid is not interrupted therefore an active α-peptide is formed, and an active ω-peptide encoded in *E. coli* JM109 genomic DNA is formed. These two peptides come together to form an active β-galactosidase enzyme, called α-complementation. Therefore X-gal is broken down to form galactose and a blue substance (5-bromo-4-chloro-indigo) (Miller, 1992) and the colonies appear blue, indicating non-recombinants.

In a recombinant, the β-galactosidase gene on the plasmid is interrupted and an inactive α-fragment is formed. An active ω-fragment encoded by *E. coli* JM109 genomic DNA is formed. However, β-galactosidase activity is not observed and the colony remains its natural white colour, indicating recombinants.

Occasionally, however, a blue colony can arise from a recombinant cell if the insert was cloned in-frame with the *lacZ* gene (if the insert does not contain an in-frame stop codon), or if the PCR process introduced mutations (Promega, 2003).

IPTG is an effective inducer of the lac operon (including *lacZ*, *lacY* and *lacA*), thereby resulting in transcription of the *lacZ*, *lacY* and *lacA* genes, but is not metabolised by β-galactosidase.

**Table 2–2.** Competent *E. coli* JM109 transformed with samples, in preparation of phase I plasmids.

| Cells transformed with | Inoculated on 2X YT* agar containing | Amount of cells inoculated | Rationale of transformation |
|---|---|---|---|
| **Controls** | | | |
| 0.1 ng undigested pKS | 100 µg/ml ampicillin, 100 µg/ml IPTG*, 100 µg/ml X-gal*. | One tenth of the cells were inoculated, in duplicate. | To determine the transformation efficiency of the competent *E. coli* JM109 cells and to test blue-white selection. |
| No DNA | 100 µg/ml ampicillin. | One tenth of the cells were inoculated. | To determine if ampicillin has degraded, or if sterile techniques were used. If growth occurred, contamination by ampicillin-resistant bacteria could have occurred, or ampicillin was degraded. |
| No DNA | no additives. | One tenth of the cells were inoculated. | To perform a viability control of the competent *E. coli* JM109 cells. |
| Positive ligation control | 100 µg/ml ampicillin, 100 µg/ml IPTG, 100 µg/ml X-gal. | Half of the cells were inoculated, in duplicate. | To determine if ligation was efficient. |
| **Experimental** | | | |
| Experimental, phase I | 100 µg/ml ampicillin, 100 µg/ml IPTG, 100 µg/ml X-gal. | Half of the cells were inoculated, in duplicate. | To determine if any phase I plasmids were constructed. |

* 2X YT – 2X yeast-tryptone; IPTG – isopropyl-β-D-thiogalactopyranoside; X-gal – 5-brom-4-chloro-3-indolyl-β-D-galactopyranoside.

## 2.2.7. Phase I plasmid extractions and restriction enzyme digestion

White colonies from the experimental plates were randomly chosen, and grown overnight in 5 ml 2X YT broth containing 100 µg/ml ampicillin (Ranbaxy), at 37°C with shaking. Plasmid DNA was extracted by alkaline lysis (Appendix B.5). The DNA was resuspended in 50 µl Tris-ethylenediaminetetraacetic acid (TE) buffer containing 8 µg deoxyribonuclease (DNase) free pancreatic ribonuclease (RNase) A (Roche), incubated for 30 min at room temperature, and quantified using λ genomic DNA standards (Appendix B.2).

Plasmid DNA as well as pKS vector DNA (~ 500 ng each) were digested with 5 U *Eco*RI and 1X SuRE/Cut Buffer H (Roche), in a final volume of 20 µl, in separate tubes.

The restriction enzyme (RE) digestion reactions were incubated at 37°C for 3.5 h. Vector pKS was RE digested as a control to determine whether the enzyme, *Eco*RI, is active. Restriction enzyme digest products were electrophoresed on a 2% agarose gel and then viewed using UV (Appendix B.1).

### 2.2.8. DNA sequencing

Recombinant plasmids were partially sequenced using the Big Dye terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and GeneAmp PCR System 9700 (Applied Biosystems) at the Department of Molecular and Cell Biology, UCT. A vector-derived primer, the M13 forward primer (Department of Molecular and Cell Biology, UCT), was used in the cycle sequencing reaction. The primer allows sequencing of the entire PCR fragment, including the primer sequences.

The DNA sequencing method uses the principle of the Sanger Dideoxy-mediated chain termination method (Stryer, 1995). Basically a PCR reaction is performed using template DNA (DNA to be sequenced), buffer, $MgCl_2$, DNA polymerase, primer, dNTPs and dideoxy nucleotides. Chain elongation is terminated when DNA polymerase incorporates a dideoxy nucleotide, because the dideoxy nucleotide lacks the 3'-hydroxyl needed for formation of the next phosphodiester bond. The chain termination method results in chains of varying lengths, with the nucleotide on the 3' side being the dideoxy nucleotide. These DNA fragments are electrophoresed on a polyacrylamide gel, with the smaller ones that electrophorese off first. Each of the four different dideoxy nucleotides is labelled with their own fluorescent dye. As they pass a detector the fluorescent labels are excited by laser. Each of the four different dideoxy nucleotides emit a different wavelength which is interpreted as a colour by the software (e.g. blue for A) and can thus be identified as either being A, C, G or T. In this way, the sequence can be determined.

### 2.2.9. Analysis of DNA sequences

Sequence results allowed the vector-derived sequences that flank the insert, to be deleted. The resultant sequences were used to perform a search on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/blast), using the 'Nucleotide-Nucleotide Basic Local Alignment Search Tool (BLAST)'. Nucleotide-Nucleotide BLAST finds similar

sequences between the one entered by the user, and those in the website's sequence database, and calculates the statistical significances of matches.

The edited sequences were aligned (using the program, DNAMAN, Lynnon BioSoft, Version 4.0) with the published *T. pallidum* (Nichols strain) 16S rRNA gene sequence in Genbank (www.ncbi.nlm.nih.gov/Genbank, accession number M88726).

## 2.3. RESULTS FOR CONSTRUCTION OF PHASE I PLASMID

### 2.3.1. PCR amplification of a region of the *T. pallidum* 16S rRNA gene

PCR was performed on different amounts of *T. pallidum* genomic DNA template, using the sense and antisense primers that target a region of the *T. pallidum* 16S rRNA gene. The results showed that a 366 bp fragment could be amplified from $10^9$, $10^7$ and $10^5$ *T. pallidum* genomic DNA equivalents (Fig. 2–4, lanes 1, 2 and 3, respectively). The bands seen below the 366 bp amplicon depict excess primers possibly in the form of primer dimers. The PCR negative control yielded no PCR amplicon (lane N, Fig. 2–4), showing that the PCR components were uncontaminated.

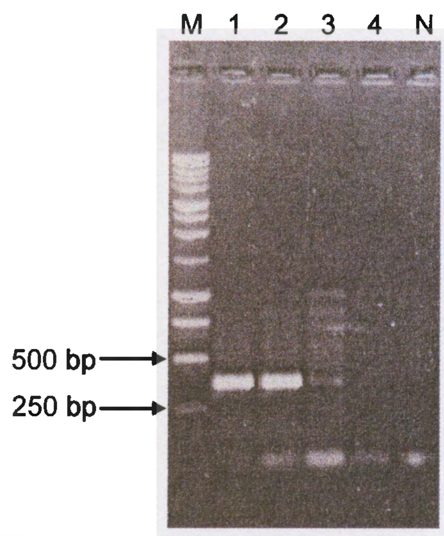



**Fig. 2–4.** Agarose-EtBr gel with PCR products obtained when using $10^9$ (lane 1), $10^7$ (lane 2), $10^5$ (lane 3) or $10^3$ (lane 4) *T. pallidum* genomic DNA equivalents and water (PCR negative control) (lane N). Lane M denotes a 1 kb DNA ladder (Promega) (Appendix C.1).

*T. pallidum* genomic DNA that had been electrophoresed on an agarose gel was not in a tight band close to the wells. Therefore it was concluded that the DNA was partially degraded, suggesting that this was the reason why no PCR product was obtained

when using $10^3$ *T. pallidum* genomic DNA equivalents (Fig. 2–4, lane 4). This genomic DNA had to be used as none other was available, and product was obtained using higher concentrations of the *T. pallidum* DNA.

It can be seen that the use of $10^9$ (Fig. 2–4, lane 1) and $10^7$ (Fig. 2–4, lane 2) *T. pallidum* genomic DNA equivalents resulted in higher amounts of 366 bp product, due to higher band intensities determined by visual inspection. The remainder of these PCR products were electrophoresed on an agarose gel, excised from the gel, purified and determined to be at a concentration of 25 ng/µl.

### 2.3.2. Cloning of the *T. pallidum* 16S rDNA internal fragment

The gel-purified 366 bp PCR product (sections 2.2.2 – 2.2.4) was ligated into the linear pGEM-T Easy Vector at 4°C. Normally when ligating cohesive DNA fragments one performs a ligation reaction at 15°C (Coyne *et al.*, 2004), however it is stated in the Promega pGEM-T and pGEM-T Easy Vector Systems handbook (2003) that to obtain a maximum number of transformants, the reactions should be incubated at 4°C (Promega, 2003). Results of the experimental ligation mix and controls that were transformed into competent *E. coli* JM109 cells (section 2.2.6) are shown in Table 2–3.

**Table 2–3.** Transformation results in preparation of the phase I plasmid.

| Cells transformed with | Results | Comments |
|---|---|---|
| **Controls** | | |
| Undigested pKS | 1 x $10^7$ cfu/µg DNA | Acceptable transformation efficiency is 1 x $10^7$ cfu/µg of plasmid DNA (Sambrook *et al.*, 1989). The cells are competent, and transformation is efficient. |
| No DNA (inoculated on 2X YT* agar) | Confluent growth | *E. coli* JM109 cells are viable. |
| No DNA (inoculated on 2X YT agar containing ampicillin) | No growth | Ampicillin is active, and sterile techniques were used. |
| Ligation control | 63% white colonies | Greater than 60% white colonies imply that the ligation had performed efficiently (Promega, 2003). |
| **Experimental** | | |
| Experimental ligation mix, phase I | 30 white colonies on each of the two plates | Recombinants were obtained. |

* 2X YT – 2X yeast-tryptone

Ten white colonies were randomly chosen from the Experimental phase I plates to confirm that they contained recombinant plasmids harbouring the 366 bp insert DNA. Plasmid DNA extracted from the 10 white colonies was subjected to *Eco*RI RE digestion (Fig. 2–5). If a plasmid with no insert was digested with *Eco*RI, one would expect a 2 997 bp (and 18 bp) linear DNA fragment; but if the 366 bp insert is present, one would expect 2 997 and 384 bp linear DNA fragments. After RE digestions with *Eco*RI, all 10 plasmids appeared to have an insert at approximately the correct size, just less than 400 bp (Fig. 2–5). Additionally, a band below the 3 000 bp linear band was observed (lanes 1 – 10, Fig. 2–5), which probably depict a form of circular, undigested plasmid, with an electrophoretic mobility similar to that of the lowest band of circular phase I plasmid (lane C2). Restriction enzyme digestion of plasmid pKS with *Eco*RI resulted in a linear band of just less than 3 000 bp in size (2 959 bp is expected) thus indicating that *Eco*RI is active.

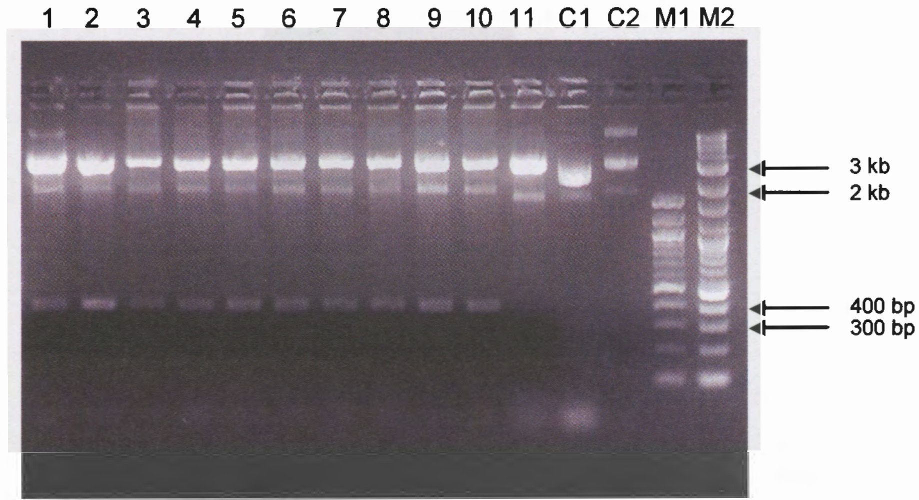



**Fig. 2–5.** Agarose-EtBr gel showing *Eco*RI profiles of the plasmids of 10 randomly chosen white clones (lanes 1 – 10), pKS-*Eco*RI control (lane 11), undigested pKS (lane C1), undigested pTPA108 (lane C2), M1 depicts 100 bp DNA ladder (New England Biolabs) (Appendix C.2) and M2 depicts 2-log DNA ladder (New England Biolabs) (Appendix C.3).

## 2.3.3. Confirmation of the presence of insert in the phase I plasmid

Two recombinant plasmids, pTPA108 and pTPA110 were randomly chosen for further experiments. (Plasmids were named as follows: For example, pTPA108, where TPA depicts *T. pallidum*, 1 depicts phase I plasmid, and the last two numbers (e.g. 08) depict the number given to the clone.) PCR amplifications (as described in section 2.2.2) on pTPA108 and pTPA110 using 5 ng plasmid DNA, confirmed that the sense and antisense primer binding sites were present on the insert DNA fragment, and that the size of the insert DNA of 366 bp is correct as there was PCR product just less than 400 bp (Fig. 2–6).

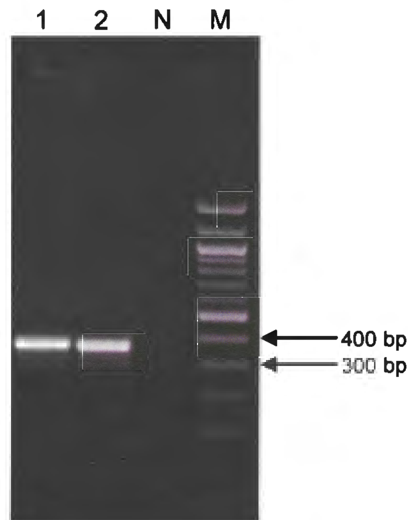



**Fig. 2–6.** Agarose (2%)-EtBr gel showing pTPA108 PCR product (lane 1), pTPA110 PCR product (lane 2), PCR negative control (N) and M depicts 100 bp DNA ladder (New England Biolabs).

### 2.3.4. Sequencing results of pTPA108 and pTPA110

Recombinant plasmids, pTPA108 and pTPA110 were partially sequenced using the vector-derived M13 forward primer. Sequencing results of pTPA108 and pTPA110 include not only the *T. pallidum* 366 bp fragment, but also the vector-derived sequences that flank the insert (since a vector-derived M13 primer was used). Hence, the vector-derived sequences were deleted from the rest of the *T. pallidum* sequence. The resultant edited sequences were blasted on the NCBI website and the first four hits were *T. pallidum* genomic DNA and the *T. pallidum* 16S rRNA gene, indicating that the insert originated from the *T. pallidum* 16S rRNA gene. Other hits included uncultured spirochete genes and 16S rRNA genes of other *Treponema* species.

Sequence results of pTPA108 and pTPA110 inserts were compared to a 366 bp portion (positions 203 – 568) of the published *T. pallidum* 16S rRNA gene sequence by multiple sequence alignment (Fig. 2–7) to confirm that the inserts were the same or similar to the *T. pallidum* 366 bp fragment.

Differences between pTPA108 and the published *T. pallidum* 16S rRNA gene sequences were as follows (Fig. 2–7):

- pTPA108 had a G at position 239, while the published sequence has an unspecified nucleoside (N) at position 239.
- pTPA108 contained a C instead of a T at position 344.
- pTPA108 contained a C instead of a G at position 549.

Differences between pTPA110 and the published *T. pallidum* 16S rRNA gene sequences were as follows (Fig. 2–7):

- The published sequence had N at position 239, while pTPA110 had a G.
- On the immediate 5' side of the antisense primer binding site there was a CCA missing.
- pTPA110 contained a C instead of a G at position 549.

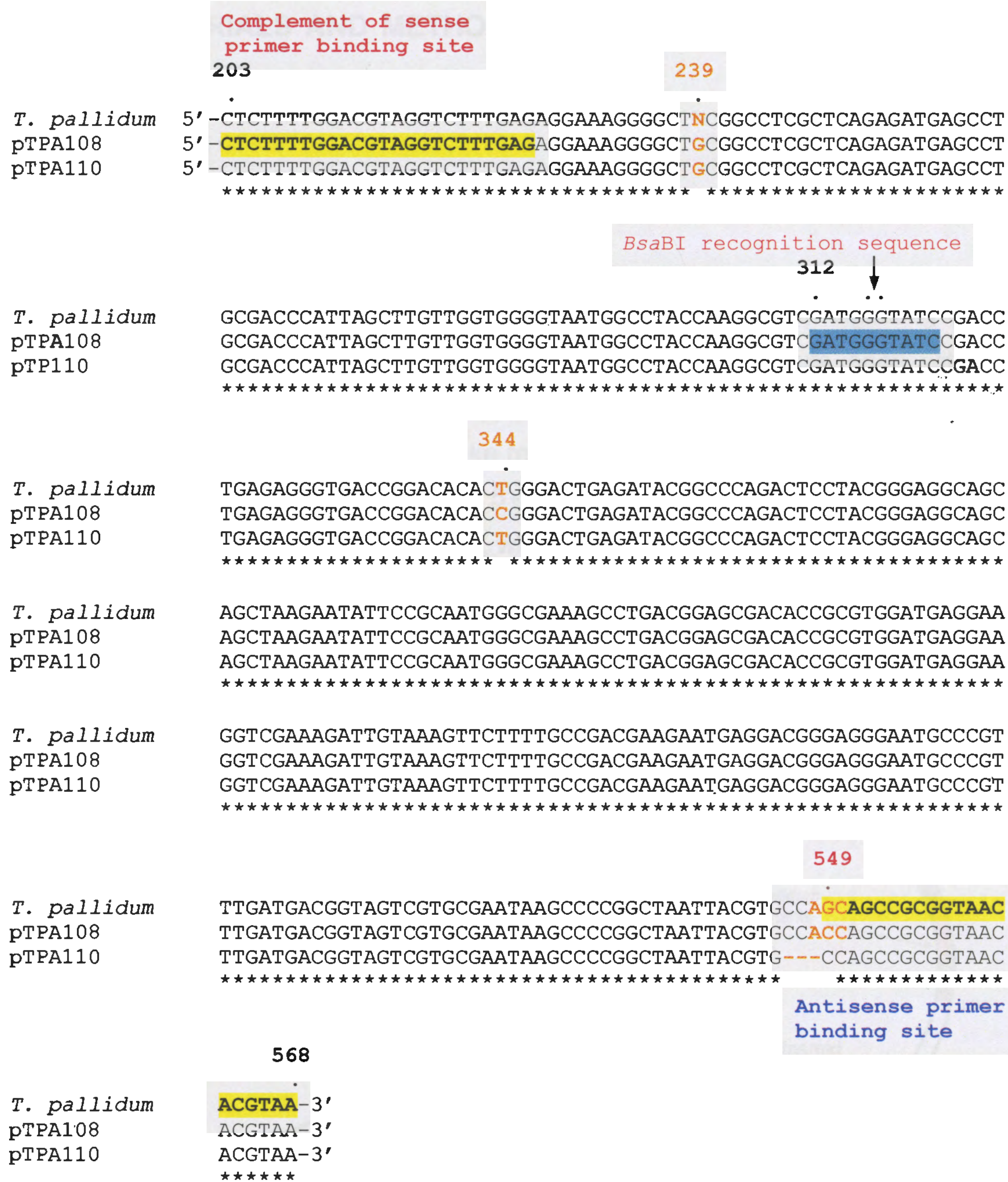



**Fig. 2–7.** Sequence alignment of a 366 bp portion of the published *T. pallidum* 16S rRNA gene sequence and inserts of pTPA108 and pTPA110 (sense strands). Numbering is according to the published sequence on Genbank (accession number, M88726). A gap indicates where the three nucleosides are different, and a * indicates where the three nucleosides are the same. The arrow indicates where *BsaBI* restricts the DNA, in order to form the positive control plasmid (section 2.4.1).

## 2.4. MATERIALS AND METHODS FOR CONSTRUCTION OF PHASE II PLASMID

### 2.4.1. Linearisation and dephosphorylation of the phase I plasmid, pTPA108

Plasmid DNA was linearised in order to insert spacer DNA, by digesting plasmid pTPA108 with *Bsa*BI RE. This enzyme was chosen as it cuts once within the *T. pallidum* 366 bp insert but not in the pGEM-T Easy Vector. Additionally, *Bsa*BI RE generates blunt ends, which would make religation of the parental vector more difficult. The recognition sequence of *Bsa*BI is 5'-GATNNINNATC-3' and it restricts between positions 316 and 317 (arrow, Fig. 2–7), which would allow for insertion of spacer DNA (Fig. 2–8).

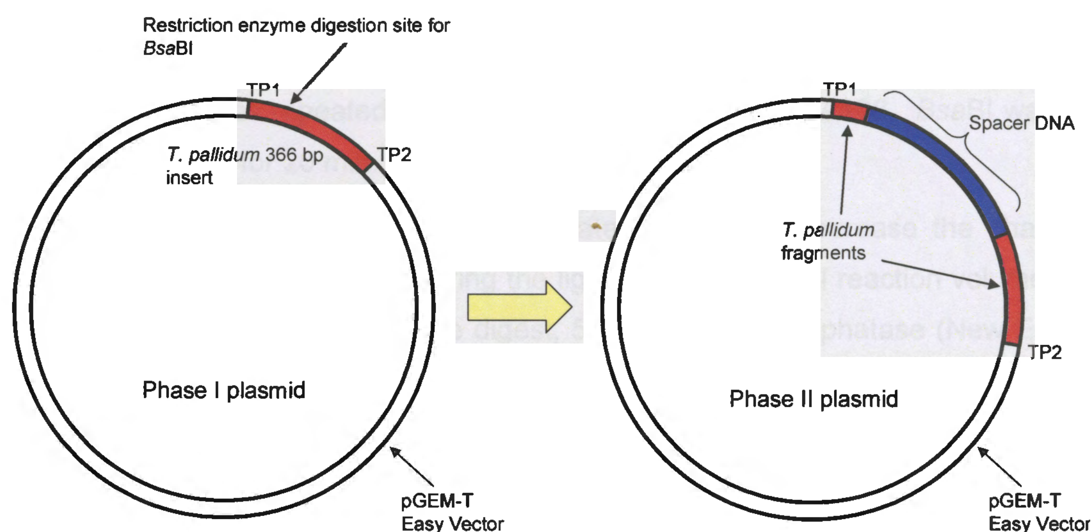



**Fig. 2–8.** Diagram showing the important components of the phase I and phase II plasmids. TP1 and TP2 depict the *T. pallidum* primer binding sites.

Approximately 4.4 µg of pTPA108 plasmid was digested using 1X NEBuffer 2 (New England Biolabs) and 5.6 U *Bsa*BI RE (New England Biolabs) at 60°C for 2 h. These digests were performed in triplicate. The RE digests (4.38 µg in each lane) were electrophoresed on a 1% agarose gel (Appendix B.1). On the other half of the gel ~ 20 ng of RE digested pTPA108 and circular pTPA108 plasmid (~ 20 ng) were electrophoresed. This half of the gel was cut off and visualised under UV irradiation to mark the position of the linearised pTPA108 band on the gel, by comparing it to circular plasmids on the half gel, and taking into account that supercoiled circular plasmid

electrophoreses fastest on an agarose gel, followed by linear and nicked circular plasmid. Alignment of the UV exposed half gel to the rest of the unexposed gel, allowed the bulk of the linearised pTPA108 band to be excised. The procedure prevented unnecessary UV exposure to the bulk sample, to prevent formation of thymine dimers, which prevent replication and gene expression until fixed (Stryer, 1995). The DNA was gel-purified according to the QIAquick Gel Extraction Kit protocol (Qiagen, 2002) and was resuspended in 30 µl 10 mM Tris-Cl, pH 8.5 (Qiagen).

In order to determine whether most of the gel-purified recombinant pTPA108 plasmid was in fact linear, a transformation (Appendix B.4) using the DNA was performed (using one hundredth of the gel-purified digested pTPA108). Controls were also performed as per Table 2–2, except for omitting 'positive ligation control'. Competent cells can only be transformed with circular DNA (in this case, the phase I plasmid, pTPA108, with the β-lactamase gene) which would then confer ampicillin resistance to the cell and allow it to grow on the ampicillin-containing media. Therefore colonies arise due to the competent cells being successfully transformed with circular plasmid DNA containing the β-lactamase gene.

The RE reaction was repeated on the gel-purified digested pTPA108. *Bsa*BI was heat inactivated at 80°C for 20 min.

Linear pTPA108 plasmid was dephosphorylated, to further decrease the chance of religation of linearised pTPA108 during the ligation step. A total reaction volume of 50 µl, using all 20 µl restriction enzyme digest, 5 U Antarctic Phosphatase (New England Biolabs) and 1X Antarctic Phosphatase Reaction Buffer (New England Biolabs) were used, and incubated at 37°C for 30 min. The dephosphorylated linear pTPA108 vector was purified according to manufacturer's instructions using the QIAquick Gel Extraction Kit (Qiagen, 2002) and eluted in 30 µl 10 mM Tris-Cl, pH 8.5 (Qiagen), and quantified using λ genomic DNA standards (Appendix B.2).

### 2.4.2. Preparation of insert spacer DNA

Genomic DNA from *E. coli* JM109 and *Staphylococcus albus* were prepared from 100 ml bacterial cultures (Appendix B.6), and then quantified on an agarose gel using λ standards (Appendix B.2).

In order to determine the combination of bacterial DNA and RE that would yield the most DNA in the 400 – 800 bp size range, genomic DNA of *Vibrio cholerae* (kindly donated by Dr L. Ah Tow, UCT), *E.coli* JM109 and *S. albus* were RE digested with

various blunt end cutters – *Bsa*BI (New England Biolabs), *Eco*RV (Roche), *Pvu*II (Roche) and *Stu*I (Roche).

Following the above, 5 µg of *E. coli* JM109 genomic DNA was digested with *Eco*RV (Roche) using 1X SuRE/Cut Buffer B (Roche) and 34 U *Eco*RV (Roche) in a total volume of 20 µl. The RE reaction was performed at 37°C for 2 h. The products were electrophoresed (Appendix B.1) on a 0.8% agarose gel with a molecular weight marker, and *Eco*RV restricted DNA fragments ranging between 400 and 800 bp were excised from the gel. Precautions were taken to avoid unnecessary UV exposure of the RE digested DNA.

Excised DNA was gel-purified according to the QIAquick Gel Extraction Kit instructions (Qiagen, 2002), and quantified using λ DNA standards (Appendix B.2). The purified DNA was concentrated using the ammonium acetate precipitation method (Appendix B.7) and resuspended in 20 µl of 10 mM Tris-Cl, pH 8.5 (Qiagen).

### 2.4.3. Ligation of linearised pTPA108 with insert spacer DNA

The spacer DNA described above (section 2.4.2) was ligated into the dephosphorylated linearised pTPA108 plasmid. Ratios of vector:insert of 4:1 and 1:1 were used, and these will be referred to as 4V:1I and 1V:1I, respectively. The ligation reaction for the 4V:1I ratio contained 1X Ligation Buffer (Roche), 10 U T4 DNA Ligase (Roche), 358 ng dephosphorylated pTPA108 DNA and 22.5 ng insert DNA; in a final volume of 20 µl. On the other hand, the 1V:1I reaction contained 224 ng dephosphorylated pTPA108 DNA and 56 ng insert DNA. As a control, 125 ng dephosphorylated linear pTPA108, without insert DNA, was incubated in the same conditions as above.

The amounts of vector and insert DNA to be used in ligation, were calculated based on the molecular weights of the DNA; the ratio of vector to insert; as well as a total DNA concentration of 10 pmol/ml that was desired (Coyne *et al.*, 2004).

### 2.4.4. Transformations using phase II plasmids

*E. coli* JM109 competent cells were transformed (Appendix B.4), with the following samples:

- Half of the experimental ligation mix (4V:1I);
- Half of the experimental ligation mix (1V:1I);
- Half of the dephosphorylated, linear, religated TPA108 ligation mix;
- 30 ng of dephosphorylated linear plasmid, pTPA108;
- 1 ng circular pKS vector;
- no DNA.

Varying amounts of *E. coli* JM109 cells were inoculated on appropriate media as shown in Table 2–4, and transformation experiments are also summarised in this table.

**Table 2–4.** Samples were transformed into competent *E. coli* JM109 cells, in preparation of phase II plasmids. Aliquots of cells equivalent to that being transformed with 30 ng vector (dephosphorylated religated linear, dephosphorylated linear, Experimental 4V:1I and 1V:1I) were inoculated, for comparability.

| **Cells transformed with** | **Ampicillin (+ or –) [a]** | **Amount of cells inoculated** | **Rationale** |
| --- | --- | --- | --- |
| **Controls** | | | |
| 1 ng circular pKS | + | One tenth of the cells were inoculated, in duplicate. | To determine the transformation efficiency of the competent *E. coli* JM109 cells. |
| deP* linear | + | Cells were centrifuged and suspended in 100 µl 2X YT* broth. All (100 µl) of the cells were inoculated (= 30 ng [b]). | To give an indication of how many plasmids are still circular. |
| deP religated linear | + | Pelleted cells were suspended in 200 µl 2X YT broth. One half (100 µl) of the cells were inoculated (= 30 ng [b]), in duplicate. | To give an indication of the amount of plasmids still circular, or linear non deP DNA. |
| No DNA | + | One tenth of the cells were inoculated. | To determine if ampicillin has degraded, or if sterile techniques were used. If growth occurred, contamination by ampicillin-resistant bacteria could have occurred, or ampicillin was degraded. |
| No DNA | – | One tenth of the cells were inoculated. | To perform a viability control of the competent *E. coli* JM109. |
| **Experimental** | | | |
| 4V:1I* | + | Cells were centrifuged and suspended in 300 µl 2X YT broth. 117 µl (= 30 ng [b]) was inoculated, and 92 µl was inoculated in duplicate. | To select for plasmids with spacer DNA insert. |
| 1V:1I* | + | Cells were centrifuged and suspended in 300 µl 2X YT broth. 150 µl (= 30 ng [b]) was inoculated, and 75 µl was inoculated in duplicate. | As above for 4V:1I. |

* 2X YT – 2X yeast-tryptone; deP – dephosphorylated; 4V:1I – ratio of vector to insert was 4:1; 1V:1I – ratio of vector to insert was 1:1.

[a] Cells were inoculated on 2X YT agar containing 100 µg/ml ampicillin (+) or no ampicillin (–).

[b] "= 30 ng" depicts the amount of cells transformed with 30 ng vector DNA.

### 2.4.5. Selection of clones, plasmid extraction and restriction enzyme digestion

Colonies were randomly taken from the 1V:1I plate, inoculated in 5ml 2X YT broth containing 100 µg/ml ampicillin (Ranbaxy), and incubated overnight on a shaker at 37°C. Plasmid DNA was extracted by alkaline lysis (Appendix B.5) and resuspended in 20 µl TE supplemented with 8 µg DNase free pancreatic RNase A (Roche) and incubated for 30 min at room temperature. Restriction enzyme digestions of the plasmids were performed using 10 U *Eco*RI (Roche), 1X SuRE/Cut Buffer H (Roche) and 5 µl plasmid DNA in a final volume of 20 µl. These RE reactions were incubated at 37°C for 2 h. Digestion products were electrophoresed on an agarose gel and visualised upon exposure to UV irradiation (Appendix B.1).

### 2.4.6. PCR on selected plasmids

Based on the results obtained from the RE digests (section 2.4.5), the plasmids indicating the presence of spacer DNA were selected for further experiments. The insert in phase II plasmids were amplified by PCR as described previously (section 2.2.2), using approximately 5 ng phase II plasmid DNA.

### 2.4.7. Optimisation of PCR on phase II plasmids

The PCR assay was performed on various templates, including circular pGEM-T Easy Vector (5 ng), *E. coli* JM109 genomic DNA (100 ng) and a phase II plasmid, pTPA238 (5 ng) (section 2.2.2) and the products were electrophoresed on an agarose gel. This was performed as part of the process of optimising the specificity of the assay.

In an attempt to increase specificity, variations to PCR were performed (Table 2–5):

- Annealing temperature of 62°C and 64°C
- Primer concentration of 0.50 and 0.25 pmol/µl
- Extension time of 1.5 and 1.0 min
- Annealing time of 1 min and 45 s
- Denaturation time of 1 min and 30 s
- $MgCl_2$ titrations were performed at 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mM

Due to suspected contamination, uncontaminated reagents from different manufacturers were used and preparation of the assay was performed in two locations of the laboratory, in an attempt to determine the source of contamination. Therefore buffers, $MgCl_2$, dNTPs and DNA polymerases from different manufacturers were used, in an effort to use uncontaminated PCR reagents (Table 2–5).

**Table 2–5.** PCR parameters were varied in PCR reactions in order to eliminate the formation of non-specific products. The parameters that were altered in each PCR assay are underlined.

| Buffer | $MgCl_2$ | DNA polymerase | dNTPs | Primers [a] | Template | PCR conditions |
|---|---|---|---|---|---|---|
| **Original conditions** | | | | | | |
| 1X Reaction Buffer (JMR Holdings) | 1.5 mM (JMR Holdings) | 1.5 U Supertherm Gold *Taq* (JMR Holdings) | 0.4 mM (ABgene) | 0.5 pmol/µl (MCB*, UCT*) | 5 ng of four selected phase II plasmids including pTPA238 | 95°C (10 min) [b]; 40 cycles of 94°C (1 min), 62°C (1 min); 72°C (1.5 min); and 72°C (5 min) |
| **Parameters varied** | | | | | | |
| 1X PCR Buffer (Takara) | 1.5 mM (Takara) | 1.5 U Supertherm Gold *Taq* (JMR Holdings) | 0.4 mM (Takara) | 0.5 pmol/µl (MCB, UCT) | 5 ng of eight selected phase II plasmids including pTPA238 | 95°C (10 min); 40 cycles of 94°C (1 min), 62°C or 64°C (1 min), 72°C (1.5 min); and 72°C (5 min) |
| 1X PCR Buffer (Takara) | 1.5 mM (Takara) | 1.5 U Supertherm Gold *Taq* (JMR Holdings) | 0.4 mM (Takara) | 0.25 or 0.5 pmol/µl (MCB, UCT) | 5 ng of pTPA238 | 95°C (10 min); 40 cycles of 94°C (1 min), 64°C (1 min), 72°C (1.5 min); and 72°C (5 min) |
| 1X PCR Buffer (Takara) | 1.5 mM (Takara) | 1.5 U Supertherm Gold *Taq* (JMR Holdings) | 0.4 mM (Takara) | 0.5 pmol/µl (MCB, UCT) | 5 ng of three phase II plasmids including pTPA238 | 95°C (10 min); 40 cycles of 94°C (1 min), 64°C (1 min), 72°C (1 or 1.5 min); and 72°C (5 min) |
| 1X PCR Buffer (Takara) | 1.5 mM (Takara) | 1.5 U Supertherm Gold *Taq* (JMR Holdings) | 0.4 mM (Takara) | 0.5 pmol/µl (MCB, UCT) | 5 ng of eight selected phase II plasmids including pTPA238 | 95°C (10 min); 40 cycles of 94°C (1 min), 64°C (1 min or 45 s), 72°C (1 min); and 72°C (5 min) |

**Table 2–5 continued.**

| Buffer | $MgCl_2$ | DNA polymerase | dNTPs | Primers [a] | Template | PCR conditions |
|---|---|---|---|---|---|---|
| 1X DyNAzyme EXT Buffer (Finnzymes) | 1.5 mM (Finnzymes) | 2 U DyNAzyme EXT (Finnzymes) | 0.4 mM (Finnzymes) | 0.5 pmol/µl (MCB, UCT) | 5 ng of pTPA238 | 94°C (2 min) [b]; 40 cycles of 94°C (1 min or 30 s), 64°C (1 min), 72°C (1 min); and 72°C (5 min) |
| 1X DyNAzyme EXT Buffer (Finnzymes) | 0.5, 1.0, 1.5, 2.0, 2.5 or 3.0 mM (Finnzymes) | 2 U DyNAzyme EXT (Finnzymes) | 0.4 mM (Finnzymes) | 0.5 pmol/µl (MCB, UCT) | 5 ng of pTPA238 | 94°C (2 min); 40 cycles of 94°C (30 s), 64°C (1 min), 72°C (1 min); and 72°C (5 min) |

* MCB – Department of Molecular and Cell Biology; UCT – University of Cape Town.

[a] Sense and antisense primers were used at equal concentrations as specified.

[b] Initial denaturation temperature and time depends on the type of DNA polymerase used, and the condition is stated in the specification sheet.

## 2.5. RESULTS FOR CONSTRUCTION OF PHASE II PLASMID

### 2.5.1. Generation of spacer DNA for the construction of phase II plasmid

When performing the *T. pallidum* diagnostic assay, the positive control RT-PCR product should ideally be 2 – 3 times the size of 366 bp in order to make the two fragments easily distinguishable. Therefore a size range of 400 – 800 bp was chosen for the spacer DNA. This would result in a positive control RT-PCR product size of between about 766 and 1166 bp.

Initial RE digestions using various combinations of genomic DNA and blunt end restriction enzymes indicated that the maximum amount of DNA in the 400 – 800 bp range was obtained when *E. coli* JM109 genomic DNA was restricted with *Eco*RV.

Based on these initial RE digestion results, a large quantity of *E. coli* JM109 genomic DNA was digested with *Eco*RV, electrophoresed on an agarose gel and the 400 – 800 bp range of the RE products was excised for gel-purification. The gel-purified spacer DNA was found to be at a concentration of 2 ng/µl and was concentrated to 40 ng/µl.

### 2.5.2. Preparation of the pTPA108 plasmid for ligation with spacer DNA

Plasmid pTPA108 was linearised with *Bsa*BI RE. Subsequent to gel-purification of pTPA108 plasmid, an aliquot was transformed into competent *E. coli* JM109 cells. A large number of transformants were obtained (5 x $10^4$ cfu/µg DNA), suggesting that a high proportion of the gel-purified pTPA238 was still circular plasmid.

The RE digestion was thus repeated on the digested pTPA108, and some product was electrophoresed on a gel together with circular pTPA108 and it appeared as if the digestion had run to completion.

### 2.5.3. Cloning of the spacer DNA into phase I plasmid, pTPA108

Ligation of dephosphorylated linearised pTPA108 with spacer DNA fragments was performed at ratios of 4V:1I and 1V:1I, and several transformations were carried out (sections 2.4.3 and 2.4.4). No IPTG and X-gal were used in the media, because all plasmids have insert (366 bp, or 366 bp + spacer DNA) in the pGEM-T Easy Vector, so all colonies would be white. Results are summarised in Table 2–6.

**Table 2–6.** These transformations were performed in preparation of a phase II plasmid.

| Cells transformed with | Transformation efficiency | Comments |
|---|---|---|
| **Controls** | | |
| Undigested pKS | 4 x $10^5$ cfu/µg DNA | This shows that the cells are competent, and transformation is occurring. However transformation efficiency is not ideal and it has decreased from 1 x $10^7$ cfu/µg DNA (see Table 2–3). |
| No DNA (inoculated on 2X YT*) | Confluent growth | *E. coli* JM109 cells are viable. |
| No DNA (inoculated on 2X YT containing ampicillin) | No growth | Ampicillin is active, and sterile techniques were used. |
| deP* linear (DL) | 2 528 cfu/µg DNA | To give an indication of how many plasmids are circular. The transformation efficiency of deP linearised phase II plasmid is relatively high, indicating that there are probably a lot of circular pTPA108 vectors in *E. coli* JM109 on the 4V:1I* and 1V:1I* Experimental plates. Vectors could be circular due to incomplete digestion. |
| deP linear religated (DLR) | 240 cfu/µg DNA | To give an indication of the amount of linear non-deP plasmids; and circular plasmids. One would expect the number of deP religated transformation efficiency to be more than that of deP linear, but it was surprisingly less. |
| **Experimental** | | |
| 4V:1I | 800 cfu/µg DNA | See section 2.6.2. |
| 1V:1I | 3 328 cfu/µg DNA | See section 2.6.2. |

* 2X YT – 2X yeast-tryptone; deP – dephosphorylated; 4V:1I – ratio of vector to insert is 4:1; 1V:1I – ratio of vector to insert is 1:1.

Plasmid DNA was extracted from 46 randomly selected colonies on the 1V:1I plate for confirmation of the presence of inserts. The plasmid DNA was digested with *Eco*RI RE; plasmids without spacer DNA, digested with *Eco*RI RE would be expected to yield the following profile: 2 997 bp and 384 bp. On the other hand, *Eco*RI RE digested plasmids harbouring spacer DNA, would be expected to result in DNA fragments of 2 997 bp; and 384 bp plus spacer DNA size.

After electrophoresing the RE fragments on an agarose gel, 16 of the 46 plasmids had spacer DNA present, indicated by the presence of RE products of 2 997 bp and greater than 400 bp (approximately 900 – 1 200 bp in size). Fig. 2–9 shows the RE digest results from a selection of the 46 plasmids. For example, lanes 1 and 2 depict phase I plasmids, only containing the 366 bp insert; whereas lanes 3, 4, 7 and 8 depict phase II plasmids with successful insertion of the digested *E. coli* JM109 DNA.

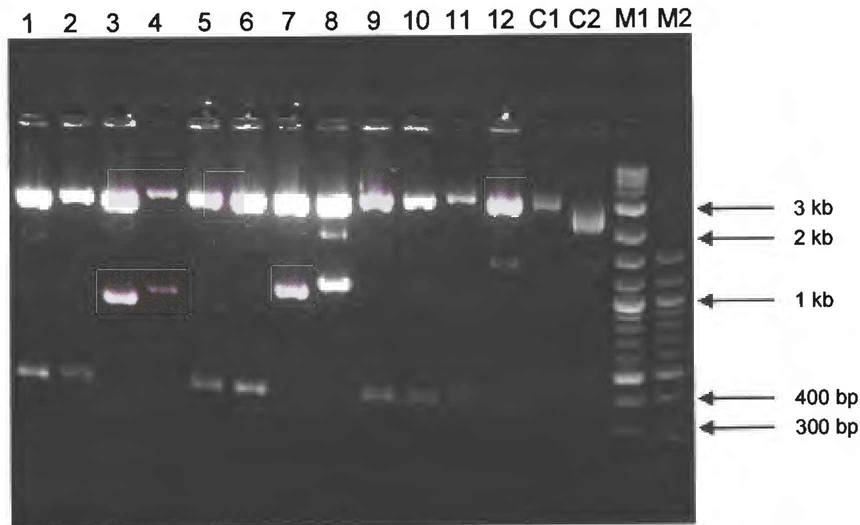



**Fig. 2–9.** Agarose (2%)-EtBr gel, where lanes 1 – 10 depict *Eco*RI RE digested products of plasmids, linear pTPA108 (11), linear pKS (12), circular pTPA108 (C1), circular pKS (C2). M1 is 2-log DNA ladder (New England Biolabs) and M2 is 100 bp DNA ladder (New England Biolabs). The above gel picture represents a subsection of the clones tested (10 of 46 randomly chosen clones).

### 2.5.4. Confirmation of primer binding sites and size of insert in phase II plasmids

Four plasmids that yielded the RE products of 2 997 bp and 900 – 1 200 bp (determined from Fig. 2–9), were selected for PCR. These plasmids correspond to the plasmids used in the RE digest i.e. lanes 3, 4, 8 and 9, respectively of Fig. 2–9. PCR was performed on these plasmids, and the product sizes, which ranged between 900 bp and 1 200 bp, were in agreement with the RE results of Fig. 2–9. The presence of a PCR product indicated the presence of primer binding sites in the phase II plasmids (Fig. 2–10).

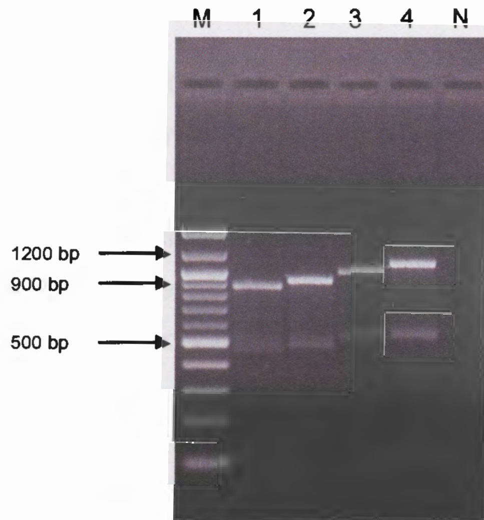



**Fig. 2–10.** PCR products using four phase II plasmids corresponding to lanes 3, 4, 8 and 9 of Fig. 2–9 (lanes 1 – 4, respectively). N depicts the PCR negative control and M depicts 100 bp DNA ladder (New England Biolabs).

## 2.5.5. Optimisation of PCR on phase II plasmids

### *2.5.5.1. <u>Results of using different parameters in PCR to eliminate non-specific bands and determine their source</u>*

When using phase II plasmids as template in PCR, in addition to the expected band on an agarose gel, a fainter band of lower molecular weight was formed during PCR (lanes 1 – 4, Fig. 2–10) and was also observed when using the remaining phase II plasmids as template in PCR reactions (data not shown). This lower molecular weight band is fainter than the intended PCR band, and will be referred to as non-specific band(s) / product(s).

Methods were employed to determine the source of the non-specific band. In order to investigate the source of the non-specific band, PCR was performed using circular pGEM-T Easy Vector or *E. coli* JM109 genomic DNA as template. However, no amplification products were detected using any of the above-mentioned templates.

In addition, various parameters were tested, as mentioned in section 2.4.7 to eliminate non-specific product formations. The variations to PCR performed did not significantly decrease the amount of the non-specific band. An annealing temperature of 64°C; extension period of 72°C (1 min); and denaturation time of 30 s may have slightly reduced the amount of the non-specific product. This is discussed fully in section 2.6.3.

#### 2.5.5.2. *Contamination*

Problems were experienced with PCR contamination wherein amplification of the negative controls gave smears when electrophoresed on agarose gels. Time was spent on determining the source of contamination in the PCR negative controls. This was performed by testing each possible contaminated component of the PCR mix in several different reactions, with the remaining components being uncontaminated; and in different locations of the laboratory. Preparing the PCR reactions in a better ventilated and sealed PCR hood, which was only available later, resolved the contamination problem.

## 2.6. DISCUSSION AND SUMMARY

### 2.6.1. Construction and sequence analysis of phase I plasmids

A 366 bp internal region of the 16S rRNA gene was amplified from *T. pallidum* genomic DNA; and ligated into the pGEM-T Easy Vector to form phase I plasmids. The inserts of two of the phase I plasmids, pTPA108 and pTPA110, were sequenced and compared to the 366 bp portion of the published *T. pallidum* 16S rRNA gene sequence. A difference between pTPA108 and the published *T. pallidum* 16S rRNA gene sequence (Fig. 2–7, section 2.3.4) included the presence of a nucleoside C instead of T at position 344. This difference probably arose since, although *Taq* polymerase has high fidelity, it does sometimes insert the wrong nucleotide. The error frequency of *Taq* polymerase is estimated to be between $2 \times 10^{-4}$ and $1.2 \times 10^{-5}$ errors per bp polymerised per cycle (Eckert & Kunkel, 1990). For both pTPA108 and pTPA110, it was discovered that nucleoside 239 is G, whereas in the database these are recorded as N (Fig. 2–7).

A difference between pTPA110 and the published *T. pallidum* 16S rRNA gene sequence included, that on the immediate 5' side of the antisense primer binding site, there was a CCA missing (Fig. 2–11). This may be because the CCA (position 546 – 548) or GCA (position 549 – 551) of *T. pallidum* genomic DNA template could have looped out and the primer bound to the remaining CCA or GCA, thereby a CCA or GCA being lost.

```
                              546
                               •
T. pallidum genomic DNA       5' CCAGCAGCCGCGGTAACACGTAA-3'
Antisense primer              3'    GGTCGGCGCCATTCTGCATT-5'
```

**Fig. 2–11.** CCA (position 546 – 548) or GCA (position 549 – 551) could have looped out and hence the sequence would be lost in the pTPA110 sequence.

It is interesting that in the sequences of both pTPA108 and pTPA110, there was C instead of G at position 549 because the antisense primer (5'-TTACGTGTTACCGCGGCTG**G**-3') bound to the sense region 5'-**G**CAGCCGCGGTAACACGTAA-3' (positions 549 – 568) of *T. pallidum* 16S rRNA gene during PCR (Fig. 2–12). The G will thus become part of the antisense sequence while the C will become part of the sense sequence, which is what is seen in the sequencing results (Fig. 2–7). It is not known why there is this discrepancy between *T. pallidum* sequence and the published antisense primer (Centurion-Lara *et al.*, 1997), i.e. why the antisense primer sequence is not 5'-TTACGTGTTACCGCGGCTG**C**-3'. The *T. pallidum* strain used in this study and that in the database are both the *T. pallidum* Nichols strain. However, it is possible that a mutation occurred within the Nichols strain used to design the primers. Alternatively, a typographical error in preparing the manuscript could have occurred; or possibly a mistake was made in constructing the primer.

```
                     549                 568
                      •                   •
T. pallidum          5'-GCAGCCGCGGTAACACGTAA-3'   Antisense primer binding site
Antisense primer     3'-GGTCGGCGCCATTGTGCATT-5'

Resulting sequences:
pTPA108              5'-CCAGCCGCGGTAACACGTAA-3'
pTPA110              5'-CCAGCCGCGGTAACACGTAA-3'
```

**Fig. 2–12.** Illustration of the mismatch between the antisense primer and *T. pallidum* template, resulting in a nucleoside change in pTPA108 and pTPA110 plasmids.

However the error arose, it is of concern since the G instead of C on the 3' end of the antisense primer would affect binding to the template (*T. pallidum* published sequence). This is of even greater concern as extension should commence from the 3' end of the primer. Surprisingly, product was formed when using *T. pallidum* genomic DNA as template.

Product was also obtained when using pTPA108 and pTPA110 as template, and the sense and antisense primers. This is understandable because of the nucleoside change of the sense sequence of the antisense primer binding sites in pTPA108 and pTPA110 plasmids, nucleoside 549 is now complementary to the 3' end of the antisense primer (Fig. 2–12). It was decided to continue using the primers that had been synthesised using the published primer sequence, given that amplification using *T. pallidum* DNA did occur successfully.

It was decided to use pTPA108 for further construction of the positive control as it was thought that the lack of CCA adjacent to the antisense primer binding site in pTPA110 would impact binding of the antisense primer. Conversely, the one nucleoside difference in pTPA108 (C instead of T) at position 344 (over 100 bases from the sense and antisense primer binding sites) is unlikely to cause a change in binding of primer to the template.

### 2.6.2. Construction of phase II plasmids

To construct the phase II plasmid, spacer DNA was generated by digesting *E. coli* JM109 genomic DNA with *Eco*RV RE. DNA fragments ranging between 400 and 800 bp were ligated into dephosphorylated linearised pTPA108. RE digestions and PCR analysis confirmed the size and presence of primer binding sites respectively, of the phase II plasmid.

Normally a greater ratio of insert to vector is used in ligations, but less insert was used in the 4V:1I reaction. In this case, the excess of insert is theoretically not needed because the dephosphorylated vector should not be able to religate to form parental vector. Additionally, one would not want concatemers of insert forming. The reaction, 1V:1I, was performed as a back-up in case dephosphorylation was not efficient.

For the transformations, in theory, the dephosphorylated linear religated (DLR) control should have yielded a higher number of transformants than the dephosphorylated (DL) linear control (Table 2–6). This is because DL transformation efficiency gives an indication of the number of circular plasmids; while DLR transformation efficiency gives an indication of the sum of circular plasmids and religated plasmid (i.e. due to the linear plasmids not being dephosphorylated). However, surprisingly, DLR yielded ~ 10X less transformants than DL. This was not investigated further, as transformants had been obtained.

Colonies were taken from the IV:1I plate and not the 4V:1I plate as it was thought that there would be fewer circular parental plasmids on the 1V:1I plate, for the following reasons:

- Following transformation with DL, approximately 3X more colonies were yielded than after ligation and transformation with 4V:1I. Therefore it was concluded that there was probably a high proportion of circular pTPA108 plasmids on the 4V:1I plate (due to incomplete digestion);
- Following the ligation reaction, transformation with 1V:1I yielded more colonies than transformation with DL. Therefore it is likely that the balance (3328 – 2528 cfu/µg DNA) of the plasmids (on the 1V:1I plate) have insert.

Hence colonies were taken from the 1V:1I plate, and plasmid DNA was extracted.

### 2.6.3. PCR on phase II plasmids and non-specific products

Plasmid pTPA238 was chosen for further experiments as it gave consistent PCR product size and the lowest molecular weight product. The reason why the latter is important is because a smaller amplicon generally amplifies better than a higher MW amplicon (Kleter *et al.*, 1998).

Besides the desired ~ 950 bp product formed during PCR using the phase II plasmid, pTPA238 as template, another product of lower molecular weight of approximately 500 bp, was formed. Although on agarose gel electrophoresis this 500 bp non-specific band is fainter than the 950 bp band, it would still not be ideal to have this non-specific band forming during the diagnostic RT-PCR assay. It may be confusing having three or more bands if a positive result is obtained, even though the non-specific fragment is distinguishable from both the control and test fragment on the basis of size. The source of this non-specific band was therefore investigated.

A possible explanation is the presence of similar, non-specific primer binding site(s) in the spacer DNA, or elsewhere, that allows non-specific binding and amplification to form the fainter, lower molecular weight DNA fragment of approximately 500 bp. Fig. 2–13 is a graphical depiction of the possible regions in which the non-specific primer binding sites possibly are, which *T. pallidum* primer binding sites would be used and the expected size of the non-specific product.

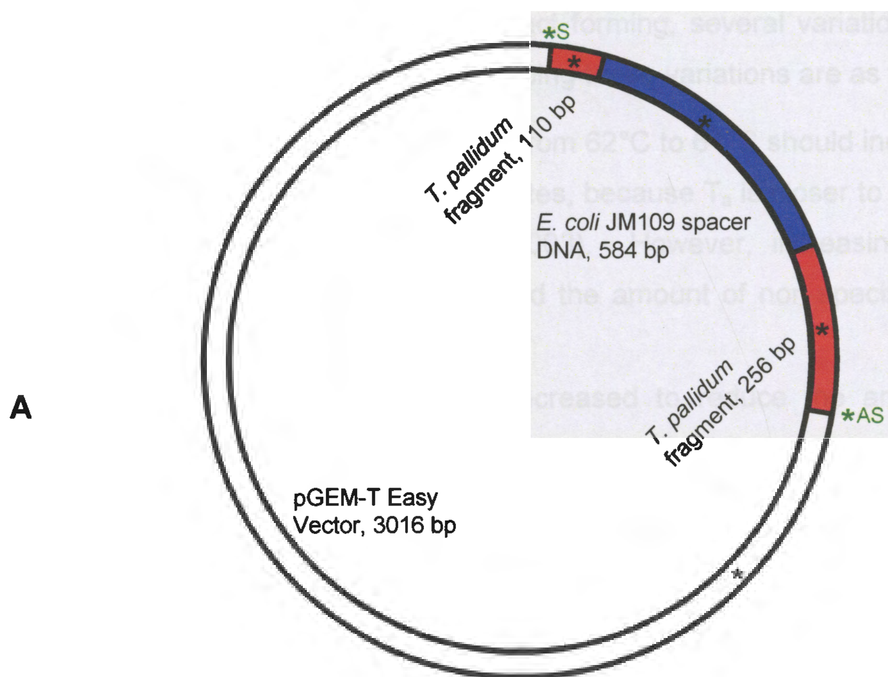
The *T. pallidum* 366 bp insert sequence was analysed for similar primer binding sites. Some primer binding sites similar to those of the intended sites were found, but the

size of the potential non-specific product was not 500 bp, the size of the non-specific product.

One of the possible non-specific primer binding sites could be within residual *E. coli* JM109 genomic DNA from the plasmid preparation. However, no product was obtained using *E. coli* JM109 genomic DNA as template in the PCR assay. Hence it can be concluded that the non-specific product is unlikely to be derived from two non-specific primer sites within residual *E. coli* JM109 genomic DNA.

The non-specific secondary primer binding site(s) could be in the pGEM-T Easy Vector or *E. coli* JM109 spacer DNA. No PCR product was formed when using circular pGEM-T Easy Vector or *E. coli* JM109 genomic DNA as template, indicating that there were not two non-specific primer binding sites within the vector or spacer DNA. If there is only one non-specific primer site within either the *E. coli* spacer DNA or pGEM-T Easy Vector, linear amplification would occur, but this product would not be detected on an agarose gel. However, the presence of one non-specific primer binding site in the vector or *E. coli* spacer DNA combined with one of the *T. pallidum* primer binding sites (within the phase II plasmid), would result in exponential amplification and products would be seen on an agarose gel. If the non-specific site is in the pGEM-T Easy Vector, the size that would be obtained would be greater than 950 bp, which does not correspond to that of the non-specific band. However, if there was one non-specific primer site within the *E. coli* spacer DNA, this combined with either the sense or antisense primer binding site could theoretically yield a product of ~ 110 – 840 bp, which is compatible with the non-specific product size of 500 bp.

A




B

| Region which may contain non-specific primer site | First primer binding site position (non-specific primer binding site) | Second primer binding site (*T. pallidum* sense or antisense primer site) | Potential size of non-specific product (bp) |
|---|---|---|---|
| *T. pallidum* 110 bp fragment | 1 – 110 | sense | 1 – 110 |
| | | antisense | 840 – 950 |
| Spacer DNA | 111 – 694 | sense | 110 – 694 |
| | | antisense | 256 – 840 |
| *T. pallidum* 256 bp fragment | 695 – 950 | sense | 694 – 950 |
| | | antisense | 1 – 256 |
| pGEM-T Easy Vector | 951 – 3 966 | sense | 950 – 3 966 |
| | | antisense | |

**Fig. 2–13. A.** Possible non-specific primer sites could be within the *E. coli* JM109 spacer DNA, pGEM-T Easy Vector or *T. pallidum* inserts. The black stars represent possible non-specific primer sites, whereas the green stars represent the intended *T. pallidum* primer sites. S – *T. pallidum* sense primer site; AS – *T. pallidum* antisense primer site.
**B.** This table depicts the potential sizes of the non-specific products obtained should the non-specific primer binding site be within that region, coupled with the *T. pallidum* sense or antisense primer binding sites.

Therefore it is likely that the non-specific product is as a result of a similar primer site in the spacer DNA combined with one of the *T. pallidum* primer binding sites.

To try to eliminate this non-specific product forming, several variations in PCR were performed (Table 2–5). The reasons for doing these variations are as follows:

- Increasing annealing temperature from 62°C to 64°C should increase specificity of primers for their target binding sites, because $T_a$ is closer to $T_m$ (Grunenwald, 2003; Hyndman & Mitsuhashi, 2003). However, increasing the annealing temperature only slightly decreased the amount of non-specific product being formed.
- The primer concentration was decreased to reduce the amount of excess primer that may have been binding non-specifically.
- Both extension time and annealing time were reduced; from 1.5 min to 1 min and 1 min to 45 s, respectively. This would reduce the amount of time available for non-specific binding. Reducing the extension time slightly decreased the amount of non-specific product being formed.
- Denaturation of 94°C for 30 s as opposed to 1 min was used, in order to keep the denaturation time as short as possible (about 30 s or less) (Finnzymes, 2005a); and also to determine whether the non-specific product amount would decrease.
- Various $MgCl_2$ concentrations were used in an attempt to increase specificity.

Unfortunately, none of these measures helped in significantly decreasing the non-specific band. Thus, although it would have been ideal to eliminate the non-specific band, this band can be differentiated from both the 366 bp band derived from *T. pallidum* and the 950 bp band derived from the positive control plasmid. The plasmid pTPA238 was used for the remainder of the project.

### 2.6.4. Summary and conclusions

This chapter describes the successful construction of a positive control plasmid, which is an important component of the *T. pallidum* diagnostic assay. Plasmid pTPA238 represents a phase II plasmid and the positive control plasmid that will be used for the RT-PCR assay. The positive control, pTPA238, of the *T. pallidum* assay would yield a product of greater size than 366 bp, in order to differentiate between the amplicon originating from *T. pallidum* and the positive control. *E. coli* harbouring this plasmid construct will be used to determine whether RNA extraction and RT-PCR are working in the assay when using clinical samples.

# 3. CHAPTER THREE – OPTIMISATION OF THE RT-PCR ASSAY

## 3.1. INTRODUCTION

The RT-PCR reaction to be used in the diagnostic assay employs the same set of primers as discussed in Chapter 2. In the RT-step, the antisense primer should bind to treponemal 16S rRNA molecules (if any are present in the sample), as well as to RNA transcribed from the positive control plasmid, pTPA238. The cDNA generated serves as template in the PCR step and exponential amplification occurs.

In order to ensure that *E. coli* TPA238 acts as a reliable positive control for RNA extraction and RT-PCR, the insert of the positive control plasmid, pTPA238, must be transcribed to RNA. This was determined in this section of the project.

Before the assay could be used on clinical samples, it needed to be optimised to be as sensitive as possible. Fewer treponemes are found during late syphilis compared to early syphilis, and syphilitic CSF may contain very low numbers of treponemes (Turner *et al.*, 1969; Burstain *et al.*, 1991).

In addition, sensitivity of PCR can be affected by inhibitors present in clinical samples and is another reason to optimise the assay. Inhibitors of PCR are numerous and include mononuclear cells, proteins, haematin (Ratnamohan *et al.*, 1998), serum, tissue components (Higuchi, 1989), IgG and heparin (Liu *et al.*, 2001). CSF also contains inhibitors and researchers have observed that these inhibitors lower the sensitivity of PCR (Hay *et al.*, 1990; Noordhoek *et al.*, 1991; Centurion-Lara *et al.*, 1997). Inhibition can occur as DNA polymerase is influenced by bacterial contaminants, concentration of $Mg^{2+}$ and factors that denature enzymes (Erlich *et al.*, 1991). Inhibition may be more likely to occur if a dilution of the clinical sample is used directly in the PCR mix (Ratnamohan *et al.*, 1998) and various strategies have been used to overcome this. In one study examining the use of PCR to detect viruses in CSF (Ratnamohan *et al.*, 1998), proteolysis and ethanol precipitation of DNA eliminated PCR inhibition. Potentially more relevant to this study, Burstain *et al.* (1991) found that inhibition occurred when boiled treponemes are added to the PCR mix. Performing alkaline lysis on treponemes with subsequent DNA extraction, removed inhibition. With these factors in mind, the process of extracting RNA from clinical samples will be optimised.

This chapter therefore describes the steps taken to optimise various facets of the diagnostic assay, including:

- Optimisation of the RNA extraction method, and RT-PCR steps with respect to the concentrations of reagents and cycling conditions;
- Determination of the sensitivity of the assay by using varying amounts of *T. pallidum* RNA in the RT-PCR assay;
- Determination of the optimal amount of *E. coli* TPA238 cells used to spike clinical samples, and the effect *E. coli* TPA238, if any, would have on the sensitivity of the assay;
- Determination of the specificity of the assay (i.e. whether the primers also amplify RNA from other species) by performing RT-PCR on RNA extracted from a number of different organisms.

## 3.2. MATERIALS AND METHODS

### 3.2.1. Templates, strains and CSF

#### *3.2.1.1. RNA templates*

*T. pallidum* RNA was kindly donated by Prof. S. Lukehart (University of Washington, USA). The quantity of *T. pallidum* RNA was expressed in amounts equivalent to a number of organisms (e.g. $10^3$ *T. pallidum* equivalents). For these RNA samples, $10^6$ *T. pallidum* equivalents is equal to approximately 18 ng of RNA (personal communication with Prof. S. Lukehart).

*Mycobacterium tuberculosis* RNA used for specificity testing was kindly donated by Ms T. Huna, UCT.

#### *3.2.1.2. Plasmid pTPA238*

Plasmid pTPA238 represents the positive control phase II plasmid (section 2.4.1, Fig. 2–8), pTPA108, linearised and ligated with spacer DNA of approximately 500 bp.

#### 3.2.1.3. Bacterial strains

*E. coli* TPA238 represents *E. coli* JM109 containing a phase II plasmid, pTPA238 (Fig. 2–8), and will be used as the positive control for the RT-PCR assay.

The following bacterial strains were used for specificity testing:

- *Escherichia coli* ATCC 25922;
- *Staphylococcus epidermidis*;
- *Staphylococcus aureus* ATCC 25923;
- *Enterococcus faecalis* ATCC 51299;
- *Pseudomonas aeruginosa* ATCC 27853;
- *Listeria monocytogenes*;
- *Streptococcus agalactiae* (Group B streptococcus);
- *Streptococcus pneumoniae*;
- *Neisseria meningitidis*;
- *Haemophilus influenzae*.

The non-ATCC strains are clinical isolates obtained from the National Health Laboratory Services diagnostic laboratory at Groote Schuur Hospital.

#### 3.2.1.4. CSF samples

In certain optimisation assays, either RNA or bacteria were spiked into CSF to simulate clinical samples more closely. The CSF used had been taken for routine diagnostic use and the supernatant was about to be discarded. The CSF samples (supernatant) were pooled and used in the optimisation assays. These CSF samples were all clinical samples that had proved to be sterile, and were CSF-FTA-ABS negative, and will be referred to as 'negative CSF'. The use of these CSF samples in optimisations of the RT-PCR assay was approved by the UCT Research Ethics Committee (REC REF 338/2005).

### 3.2.2. Optimisation of the sensitivity of the RT-PCR assay

#### 3.2.2.1. Baseline RT-PCR conditions

Prior to the optimisation steps an RT-PCR assay was performed. RT-PCR reactions contained: 1X RobusT Reaction Buffer (Finnzymes), 1.5 mM $MgCl_2$ (Finnzymes), 10 U

M-MuLV Reverse Transcriptase RNase $H^-$ (Finnzymes), 2 U DyNAzyme EXT DNA Polymerase (Finnzymes), 0.2 mM each dNTP (Finnzymes), 8 mM dithiothreitol (DTT) (Sigma-Aldrich), 50 U RiboLock RNase Inhibitor (Fermentas), 0.2 pmol/µl each of sense and antisense primers (Department of Molecular and Cell Biology, UCT) in a 50 µl reaction. A master mix containing these components was prepared, and then 49 µl aliquoted to PCR tubes. Subsequently, RNA (1 µl) was added to the tubes except for the negative control. In all RT-PCR reactions, a negative control was included in which water instead of RNA was added. Nuclease free water (MP Biomedicals) was used in all RT-PCR reactions described in this thesis.

The cycling conditions employed consisted of an RT-step at 42°C (50 min) followed by denaturation at 94°C (2 min) and then 40 cycles of denaturation at 94°C (30 s), annealing at 64°C (1 min) and elongation at 72°C (1 min). A final elongation step at 72°C (5 min) was included. The RT-PCR reactions were performed in a Thermal Cycler 2720 (Applied Biosystems).

Primer, dNTP, reverse transcriptase and DNA polymerase concentrations used were as per the RT-PCR start-up recommendations (Finnzymes, 2005b). The RT-step was performed at 42°C for 50 min as this is the average temperature and time recommended in the RT-PCR kit manual (Finnzymes, 2005b). This RT condition also applies to other reverse transcriptase enzymes (e.g. Superscript II, Invitrogen) and was described by Centurion-Lara *et al.* (1997).

#### 3.2.2.2. Optimisation of the RT-PCR reaction

Various alterations in the RT-PCR parameters were performed, as detailed in Table 3–1, in order to optimise the sensitivity of the assay. Each optimisation was performed at least twice. The parameters that were investigated were:

- Annealing temperature of 62°C and 64°C
- Various primer (0.2 – 0.5 pmol/µl) and $MgCl_2$ concentrations (1.5 – 3.0 mM)
- Various dNTP (0.2 – 0.4 mM) and $MgCl_2$ concentrations (1.5 – 3.0 mM)
- Various DTT concentrations (0 – 8 mM)
- Elongation step of 1.0 min and 1.5 min
- Increasing amounts of DNA polymerase from 2 – 4 U
- Increasing amounts of reverse transcriptase from 10 – 20 U

- Heat denaturation of RNA prior to the RT-step: RNA, added to primers and water, was heat denatured at 94°C for 1 min and then kept on ice for at least 1 min to cool. The remaining components of the RT-PCR mix were then added. For samples that were not heat denatured, RNA, primers and water were kept on ice until the remaining RT-PCR ingredients were added.

All reagents used in optimisations were as per section 3.2.2.1. Items that remained constant throughout the optimisation process were: 1X RobusT Reaction Buffer (Finnzymes), and 50 U RiboLock RNase Inhibitor (Fermentas).

**Table 3–1.** Table showing the experiments performed to optimise the sensitivity of the RT-PCR assay, using *T. pallidum* RNA as template. The parameters that were varied in each experiment are underlined. Each optimisation was performed at least twice.

| $MgCl_2$ | dNTPs | DTT | Primers [a] | Reverse transcriptase | DNA polymerase | *T. pallidum* RNA equivalents | Heat denaturation | RT-PCR conditions |
|---|---|---|---|---|---|---|---|---|
| 1.5 mM | 200 µM | 8 mM | 0.2 pmol/µl | 10 U | 2 U | $10^5$, $10^4$, $10^3$, $10^2$ | No | 42°C (50 min); 94°C (2 min); 40 cycles of 94°C (30 s), 64°C or 62°C (1 min), 72°C (1 min); 72°C (5 min) |
| 1.5, 2.0, 2.5, 3.0 mM | 200 µM | 8 mM | 0.2, 0.3, 0.4, 0.5 pmol/µl | 10 U | 2 U | $10^5$ | No | 42°C (50 min); 94°C (2 min); 40 cycles of 94°C (30 s), 62°C (1 min), 72°C (1 min); 72°C (5 min). |
| 1.5, 2.0, 2.5, 3.0 mM | 200, 400 µM | 8 mM | 0.5 pmol/µl | 10 U | 2 U | $10^5$ | No | 42°C (50 min); 94°C (2 min); 40 cycles of 94°C (30 s), 62°C (1 min), 72°C (1 min); 72°C (5 min). |
| 2 mM | 400 µM | 0, 2, 4, 6, 8 mM | 0.5 pmol/µl | 10 U | 2 U | $10^3$, $10^2$, $10^1$ | No | 42°C (50 min); 94°C (2 min); 40 cycles of 94°C (30 s), 62°C (1 min), 72°C (1 min); 72°C (5 min). |
| 2 mM | 400 µM | 0 mM | 0.5 pmol/µl | 10 U | 2 U | $10^3$, $10^2$, $10^1$ | No | 42°C (50 min); 94°C (2 min); 40 cycles of 94°C (30 s), 62°C (1 min), 72°C (1 or 1.5 min); 72°C (5 min). |

**Table 3–1 continued.**

| $MgCl_2$ | dNTPs | DTT | Primers [a] | Reverse transcriptase | DNA polymerase | *T. pallidum* RNA equivalents | Heat denaturation | RT-PCR conditions |
|---|---|---|---|---|---|---|---|---|
| 2 mM | 400 μM | 0 mM | 0.5 pmol/μl | 10, 15, 20 U | 2 U | $10^3$, $10^2$, $10^1$ | No | 42°C (50 min); 94°C (2 min); 40 cycles of 94°C (30 s), 62°C (1 min), 72°C (1.5 min); 72°C (5 min). |
| 2 mM | 400 μM | 0 mM | 0.5 pmol/μl | 10 U | 2, 3, 4 U | $10^3$, $10^2$, $10^1$ | No | 42°C (50 min); 94°C (2 min); 40 cycles of 94°C (30 s), 62°C (1 min), 72°C (1.5 min); 72°C (5 min). |
| 2 mM | 400 μM | 0 mM | 0.5 pmol/μl | 10 U | 2 U | $10^3$, $10^2$, $10^1$ | Heat denaturation or no heat denaturation | 42°C (50 min); 94°C (2 min); 40 cycles of 94°C (30 s), 62°C (1 min), 72°C (1.5 min); 72°C (5 min). |

[a] The sense and antisense primers were used at equal concentrations, as specified.

### 3.2.3. Methods used in the evaluation of the positive control, *E. coli* TPA238

#### *3.2.3.1. Quantification of* E. coli *TPA238*

In order to quantify the number of cells to be used for the RNA extraction method using the RNeasy column (Qiagen), absorbance relating to a certain number of cells was determined. Absorbance of each sample was measured at 600 nm using a Biomate 5 spectrophotometer (Thermo Electron Corporation). Five ml of 2X YT broth supplemented with 100 µg/ml ampicillin (Ranbaxy) was inoculated with *E. coli* TPA238, and incubated overnight at 37°C on a shaker. The culture was diluted to a final absorbance of approximately 0.05 in 100 ml 2X YT broth supplemented with 100 µg/ml ampicillin (Ranbaxy), and incubated at 37°C on a shaker. Every hour an aliquot of the culture was removed from the culture flask to measure its absorbance at 600 nm. Serial 10-fold dilutions of the aliquot were made in saline before inoculating 100 µl volumes on 2X YT agar containing 100 µg/ml ampicillin (Ranbaxy). Plates were incubated overnight at 37°C, after which colonies were counted. Inoculation was carried out at least six times for each time point. Taking into account the number of colonies on the plate, dilutions and volumes inoculated, absorbance could be related to cfu/ml. The absorbance relative to cfu/ml was confirmed by performing the procedure again, in duplicate, and inoculating around the time points when the culture reached $10^7$ to $10^9$ cells/ml.

#### *3.2.3.2. RNA extractions*

*E. coli* TPA238 was grown as previously described (section 3.2.3.1). When the required absorbance was reached, the cells were harvested for RNA extraction (Appendix B.8). RNase precautions were observed (Appendix B.9).

#### *3.2.3.3. Agarose gel electrophoresis*

The integrity of the RNA was determined by electrophoresis on a 1.5% agarose-TAE gel at 4V/cm, using precautions as per Appendix B.9. To determine that the gel did indeed contain RNA, an agarose-TAE gel was incubated with 2.5 mg DNase free pancreatic RNase A (Roche), in 100 ml 1X TAE buffer, for ~ 4.5 h.

### 3.2.3.4. *DNase I treatment*

RNA was DNase I treated in a reaction containing 1X DNase I Reaction Buffer (New England Biolabs), 4 U RNase-free DNase I (New England Biolabs), 100 U RiboLock RNase Inhibitor (Fermentas) and made up to 100 µl with RNase-free water (MP Biomedicals). This was incubated at 37°C for 10 min. After the reaction had been completed, the DNase I was heat inactivated at 75°C for 10 min. Since RNA is hydrolysed if divalent cations are present during heat inactivation of the DNase I, EDTA (final concentration of 5 mM) was added for chelation of $Mg^{2+}$ (Wiame *et al.*, 2000), which is present in the DNase I Reaction Buffer.

### 3.2.3.5. *PCR*

PCR reactions were performed under the following conditions: 1X DyNAzyme EXT Buffer (Finnzymes), 2 mM $MgCl_2$ (Finnzymes), 0.4 mM dNTPs (Finnzymes), 2 U DyNAzyme EXT DNA Polymerase(Finnzymes), 0.5 pmol/µl each of sense and antisense primers (Department of Molecular and Cell Biology, UCT) in a 50 µl reaction. Cycling conditions used were 94°C (2 min); 40 cycles of 94°C (30 s), 62°C (1 min), 72°C (1.5 min); 72°C (5 min), using a Thermal Cycler 2720 (Applied Biosystems).

## 3.2.4. Optimisation of preparation and use of RNA for the diagnostic RT-PCR assay

### 3.2.4.1. *Use of both* T. pallidum *and* E. coli *TPA238 in RT-PCR*

When both *E. coli* TPA238 and *T. pallidum* RNA were used in the same RT-PCR reactions, RNA was extracted from 2.5 x $10^3$, 5 x $10^3$, 7.5 x $10^3$ and 1 x $10^4$ *E. coli* TPA238 (Appendix B.10). Half (15 µl) of the extracted *E. coli* TPA238 RNA was used in RT-PCR reactions, and *T. pallidum* RNA was added to the same RT-PCR reaction.

### 3.2.4.2. *Preparation of RNA from simulated CSF clinical samples*

*E. coli* TPA238 was spiked in 1.5 ml pooled CSF and RNA was extracted, with on-column DNase digestion (Appendix B.10). This procedure was followed to mimic what will happen to clinical samples. A 1.5 ml volume of the pooled negative CSF was used as it was thought that this would be the maximum clinical sample volume used for

evaluation of the RT-PCR assay, and hence would show a more accurate inhibitory effect compared to using a lower volume.

### *3.2.4.3. RNA precipitation tests*

In an attempt to increase RNA yield from the clinical samples, various RNA precipitation methods were tested as described below. For further explanation see section 3.4.5. Each precipitation method was performed at least twice. Since CSF clinical samples were stored at –70°C, pooled CSF that had been spiked with $10^4$ *T. pallidum* RNA, was frozen at –70°C for at least 16 h, before being thawed.

#### *i. Sodium acetate (NaAc) precipitation of RNA*

This method was adapted from Coyne *et al.*, 2004 and was performed to determine whether the method works when using CSF. Frozen CSF (1.5 ml) containing $10^4$ *T. pallidum* equivalents of RNA, was thawed in a fridge. Then, 0.1 volume 3M NaAc, pH 5.2 was added. Two volumes of cold ethanol (EtOH) was added to precipitate RNA (Ausubel *et al.*, 1987), and 20 µg of glycogen (Roche) per ml of dilute RNA solution was added to aid in precipitation of RNA (Ausubel *et al.*, 1987; Coyne *et al.*, 2004). The mixture was incubated at –20°C overnight to precipitate RNA, and centrifuged at 14 000 rpm for 15 min at 4°C to pellet RNA. The supernatant fluid (SNF) was removed. One ml of 70% cold EtOH was added to the pellet, and the mixture was vortexed. This was centrifuged at 14 000 rpm for 15 min at 4°C, and the SNF was removed. Addition of 70% ethanol and centrifugation was performed in order to remove salts (Ausubel *et al.*, 1987). The pellet was then air-dried. RNA was suspended in 100 µl TE, pH 8, and 350 µl RNeasy Mini Kit (Qiagen) lysis buffer, RLT. Ethanol (250 µl) was added and then the mix was transferred to the RNeasy column. Subsequently, RNA was cleaned and eluted, following Appendix B.8 starting at step 6. RNA precipitation using CSF was performed four times.

The same procedure was followed, using 1.5 ml saline instead of CSF. These saline controls were performed in order to determine whether component(s) of the CSF were interfering with RNA precipitation, by comparing to CSF.

### *ii. Proteinase K treatment and NaAc precipitation of RNA*

This method was adapted from New England Biolabs and Finnzymes proteinase K package inserts, and involved proteinase K treatment to degrade protein. Frozen CSF (1.5 ml) containing $10^4$ *T. pallidum* equivalents of RNA was thawed in a fridge. Subsequently, 150 µg of proteinase K (Finnzymes) was added and the mixture was incubated at 37°C for 40 min. After that, 0.1 volume 3M NaAc, pH 5.2, 1 volume cold isopropanol (to precipitate RNA, Ausubel *et al.*, 1987), and 20 µg of glycogen (Roche) per ml of dilute RNA solution, were added. This was incubated at –70°C for 30 min to precipitate RNA, and then centrifuged at 14 000 rpm for 20 min at 4°C, and the SNF was removed. One ml of 70% cold EtOH was added to the pellet and the mixture was vortexed. This was centrifuged at 14 000 rpm for 15 min at 4°C, the SNF was removed and the pellet was air-dried. RNA was suspended in 100 µl TE, pH 8, and 350 µl RLT, and 250 µl EtOH was added, and transferred to the RNeasy column. As described in section 3.2.4.3.i, subsequent steps are described in Appendix B.8, from step 6.

As a control and for comparison, the same procedure was followed as above, except that no proteinase K was used.

### *iii. Ammonium acetate ($NH_4OAc$) precipitation with β-mercaptoethanol addition*

Five µl of β-mercaptoethanol was added to 1.5 ml frozen CSF containing $10^4$ *T. pallidum* RNA equivalents, and was then thawed in a fridge. This volume of β-mercaptoethanol was determined according to the RNeasy Mini Kit amount for RLT (Qiagen, 2001). The rest of the procedure was the same as 3.2.4.3.i except that 5 M $NH_4OAc$ (Ambion) was used and the remainder of β-mercaptoethanol (11.5 µl) was added when glycogen was added. β-mercaptoethanol was added to try and denature protein as it may have interfered with RNA precipitation.

As a control and for comparison, the same procedure was followed as above, except that no β-mercaptoethanol was used.

### *iv. Protein removal and $NH_4OAc$ precipitation of RNA*

This method was adapted from Coyne *et al.*, 2004 and Fermentas glycogen package insert. Frozen CSF (1.5 ml) containing $10^4$ *T. pallidum* equivalents of RNA was thawed in a fridge, and 1 volume 5 M $NH_4OAc$ (Ambion) was added and incubated at room

temperature for 30 min. This was then centrifuged at 14 000 rpm for 15 min at 10°C, the SNF was transferred to a new tube and any protein should be in the pellet. One volume cold isopropanol and 20 µg of glycogen per ml of dilute RNA solution (Roche) were added to the SNF, and incubated at –70°C for 1 h. The protocol of section 3.2.4.3.i was then followed starting at the first centrifugation step.

A second method was performed as a control, to compare to the first method, and was adapted from Ambion and Fermentas glycogen package inserts. Frozen CSF (1.5 ml) containing $10^4$ *T. pallidum* equivalents of RNA was thawed in a fridge. Then, 0.1 volume 5M $NH_4OAc$ (Ambion), 1 volume cold isopropanol, and 20 µg of glycogen (Roche) per ml of dilute RNA solution was added. This was incubated at –70°C for 1 h. The protocol of section 3.2.4.3.i was then followed starting at the first centrifugation step.

### *v. Simulation of clinical samples*

This method was adapted from Centurion-Lara *et al.*, 1997. CSF volumes of 500 µl were now used to better mimic what would happen to the clinical samples to be used in the preliminary evaluation described in this thesis, should RNA precipitation be used, as information was obtained that the average volume of these CSF clinical samples was 500 µl. CSF (500 µl) was spiked with $10^4$ *T. pallidum* RNA, frozen and then thawed in a fridge. Following this, 5 x $10^3$ *E. coli* TPA238, 0.1 volume 3 M $NH_4OAc$ (Ambion), 2.5 volumes cold EtOH, and 20 µg of glycogen (Roche) per ml of dilute RNA solution glycogen were added. This was centrifuged at 14 000 rpm for 30 min at 4°C and the SNF was removed. Subsequently, RNA was extracted, cleaned and eluted as described in Appendix B.10, starting at step 3.

## 3.3. RESULTS

### 3.3.1. Optimisation of the sensitivity of the RT-PCR reaction

In order to increase sensitivity of the RT-PCR reaction, various parameters were tested as described below. The relative amount of product in these optimisations was determined by judging the intensities of the bands on the agarose-EtBr gel, as well as by determining the lowest amount of *T. pallidum* RNA that could be detected when changing parameters.

#### 3.3.1.1. *Annealing temperature*

In order to optimise the assay, annealing temperatures of 62°C or 64°C were used in the RT-PCR reactions. Amplicons were detected when RT-PCR was performed on $10^2$ *T. pallidum* equivalents of RNA using a $T_a$ of 62°C (lane 7, Fig. 3–1), but not when a $T_a$ of 64°C was used (lane 8). In addition, more product (as evidenced by a brighter DNA band) was obtained when using $T_a$ of 62°C as can be seen in Fig. 3–1, lane 1 (62°C) vs. lane 2 (64°C); and lane 3 (62°C) vs. lane 4 (64°C). Therefore $T_a$ of 62°C was used in future reactions.

**A**

| **Lane number** | ***T. pallidum* RNA** | **Annealing temperature** |
|---|---|---|
| **1** | $10^5$ | 62°C |
| **2** | | 64°C |
| **3** | $10^4$ | 62°C |
| **4** | | 64°C |
| **5** | $10^3$ | 62°C |
| **6** | | 64°C |
| **7** | $10^2$ | 62°C |
| **8** | | 64°C |
| **Negative control** | none | 62°C |

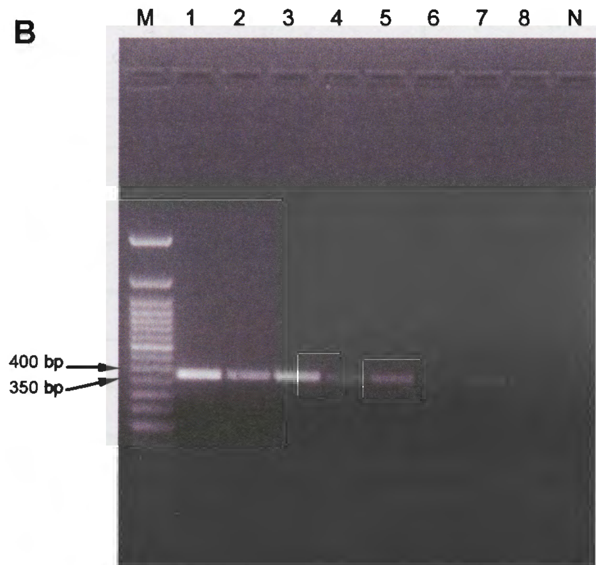



**Fig. 3–1. A.** The combinations of RNA amounts and annealing temperatures used. **B.** Agarose-EtBr gel showing RT-PCR products, with numbers corresponding to those in panel A, N is the negative control using water, and M is a 50 bp DNA ladder (New England Biolabs) (Appendix C.4).

### *3.3.1.2. Primer and $MgCl_2$ titrations*

In order to increase sensitivity, the assay was evaluated using different combinations of primer and $MgCl_2$ concentrations. Primer concentration can influence sensitivity since insufficient primer could render the assay less sensitive (Grunenwald, 2003). Various $MgCl_2$ concentrations were also used, as free $Mg^{2+}$ is required by DNA polymerase to function, and is also chelated by primers (Grunenwald, 2003).

The amount of RT-PCR product increased as the primer concentrations increased to 0.4 and 0.5 pmol/µl of each primer (Fig. 3–2). If one compares lanes 1 – 4 to lanes 13 – 16 of Fig. 3–2, brighter bands (indicating more amplification) are visible in the latter four bands when 0.5 pmol/µl of primers were used. Increasing primer concentration from 0.4 – 0.5 pmol/µl did not detrimentally affect product amount and since this primer concentration was successfully used in the PCR assay (section 2.2.2), 0.5 pmol/µl was used in the RT-PCR assay. When comparing lanes 1 and 2, a slightly brighter DNA band is visible in lane 2, corresponding to 2 mM $MgCl_2$ in the reaction mixture. Non-specific bands of higher MW were observed when $MgCl_2$ concentrations of 2.5 and 3.0 mM were used, as can be seen in lanes 8, 12, 15 and 16 of Fig. 3–2. Therefore 2 mM $MgCl_2$ was used in future RT-PCR reactions.

**A**

| Lane number | $MgCl_2$ concentration (mM) | Primers (pmol/µl) [a] |
|---|---|---|
| 1 | 1.5 | 0.2 |
| 2 | 2.0 | |
| 3 | 2.5 | |
| 4 | 3.0 | |
| 5 | 1.5 | 0.3 |
| 6 | 2.0 | |
| 7 | 2.5 | |
| 8 | 3.0 | |
| 9 | 1.5 | 0.4 |
| 10 | 2.0 | |
| 11 | 2.5 | |
| 12 | 3.0 | |
| 13 | 1.5 | 0.5 |
| 14 | 2.0 | |
| 15 | 2.5 | |
| 16 | 3.0 | |
| Negative control | 3.0 | 0.5 |

[a] The sense and antisense primers were used at equal concentrations, as specified.

**B**

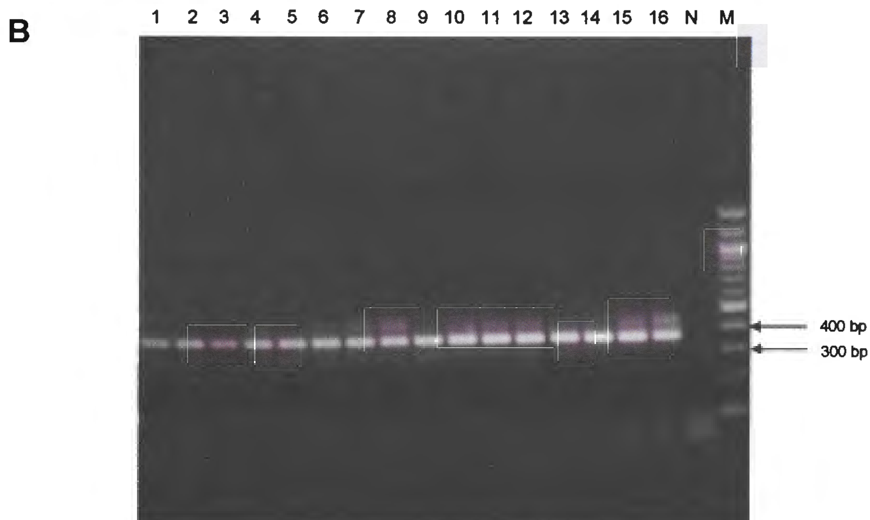



**Fig. 3–2. A.** Various primer and $MgCl_2$ concentrations were used. Note that $10^5$ *T. pallidum* equivalents of RNA were used for each reaction; and no RNA was used for the negative control. **B.** Agarose-EtBr gel showing RT-PCR amplicons when using various primer and $MgCl_2$ concentrations. Numbers correspond to those in panel A. N depicts the RT-PCR negative control and M is a 100 bp DNA ladder (New England Biolabs).

### 3.3.1.3. *dNTP and $MgCl_2$ titrations*

In a similar fashion as described with the primers (section 3.3.1.2) the assay was also evaluated with different concentrations of dNTPs and $MgCl_2$. The dNTP concentration would influence the sensitivity of the assay, as insufficient dNTPs would render the assay less sensitive. On the other hand, higher concentrations of dNTPs can negatively affect product yield and specificity (Grunenwald, 2003). Deoxynucleotides chelate $MgCl_2$ (Grunenwald, 2003), so $MgCl_2$ titrations were also performed. Using 400 µM rather than 200 µM dNTPs increased sensitivity as can be seen when comparing lanes 2 and 6, Fig. 3–3. Therefore 400 µM dNTPs were used from this point on. Using 400 µM dNTP together with 2.5 or 3 mM $MgCl_2$ resulted in non-specific bands – lanes 7 and 8 have non-specific bands whereas lane 6 (2 mM $MgCl_2$) does not. When the reaction mixture contained 1.5 mM $MgCl_2$ (lane 5) less product was visible than if it contained 2 mM $MgCl_2$ (lane 6). Therefore, taking into account sensitivity and specificity, 2 mM $MgCl_2$ was used for future RT-PCR reactions.

**A**

<table>
<tr><th>Lane number</th><th>MgCl<sub>2</sub> concentration (mM)</th><th>dNTP (µM)</th></tr>
<tr><td>1</td><td>1.5</td><td rowspan="4">200</td></tr>
<tr><td>2</td><td>2.0</td></tr>
<tr><td>3</td><td>2.5</td></tr>
<tr><td>4</td><td>3.0</td></tr>
<tr><td>5</td><td>1.5</td><td rowspan="4">400</td></tr>
<tr><td>6</td><td>2.0</td></tr>
<tr><td>7</td><td>2.5</td></tr>
<tr><td>8</td><td>3.0</td></tr>
<tr><td>Negative control</td><td>3.0</td><td>400</td></tr>
</table>

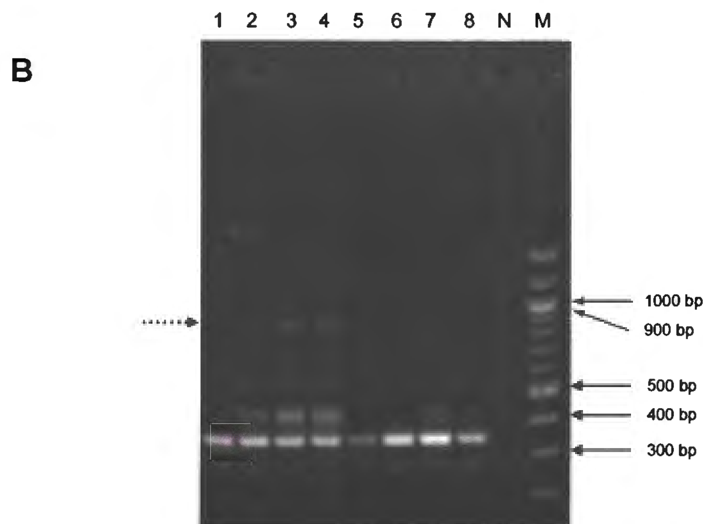



**Fig. 3–3. A.** Varying dNTP and $MgCl_2$ concentrations are shown. RT-PCR reactions contained $10^5$ *T. pallidum* RNA equivalents and the negative control contained no RNA.
**B.** Agarose-EtBr gel depicting RT-PCR products when performing dNTP and $MgCl_2$ titrations, using $10^5$ *T. pallidum* RNA equivalents. Numbers correspond to those in panel A, N is the RT-PCR negative control and M is a 100 bp DNA ladder (New England Biolabs).

Two higher MW non-specific bands were observed in lanes 3 and 4, Fig. 3–3. The higher MW band (dotted arrow, Fig. 3–3) could possibly be the same size as the RT-PCR product when using *E. coli* TPA238 RNA. Therefore, the amplification products of lanes 3 and 4, and the product obtained when performing RT-PCR on RNA from *E. coli* TPA238 were electrophoresed on a gel in adjacent wells. The non-specific band was found to be of a lower MW than the amplification product from *E. coli* TPA238 (~ 950 bp) (data not shown). Hence, this non-specific band (dotted arrow, Fig. 3–3) would not present a problem in interpretation of the assay. Additionally, it is unlikely to be a problem as these conditions were not used in the assay.

#### 3.3.1.4. Various DTT concentrations

Normally, DTT is required by an RNase inhibitor to function (Sambrook *et al.*, 1989). However, the concentration of DTT was not specified in the RiboLock RNase Inhibitor manual, and therefore this was empirically confirmed. It appears that using no DTT results in the most amount of RT-PCR product. When no DTT was used in the reaction mixture (Fig. 3–4, lanes 1, 6 and 11) the intensities of the bands are brighter than when DTT was used. This is particularly evident when $10^1$ *T. pallidum* RNA equivalents were used as template (lane 11 with no DTT vs. lanes 12 – 15 with increasing concentrations of DTT). Hence, DTT was omitted from subsequent RT-PCR reactions.

**A**

| Lane number | *T. pallidum* RNA | DTT (mM) |
|---|---|---|
| **1** | | 0 |
| **2** | | 2 |
| **3** | $10^3$ | 4 |
| **4** | | 6 |
| **5** | | 8 |
| **6** | | 0 |
| **7** | | 2 |
| **8** | $10^2$ | 4 |
| **9** | | 6 |
| **10** | | 8 |
| **11** | | 0 |
| **12** | | 2 |
| **13** | $10^1$ | 4 |
| **14** | | 6 |
| **15** | | 8 |
| **Negative control** | none | 8 |

**B**

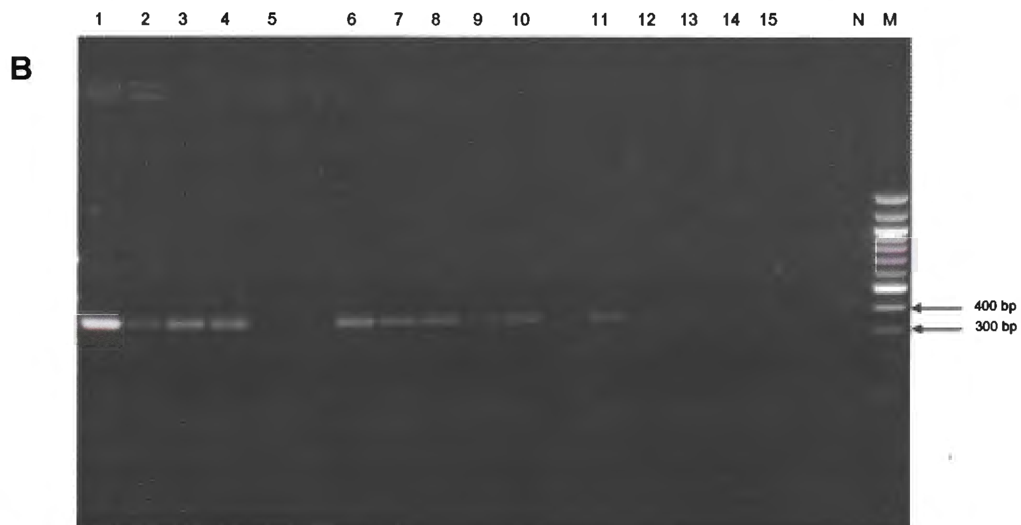



**Fig. 3–4. A.** Various combinations of DTT concentrations together with the RNA amounts used, are shown.
**B.** Agarose-EtBr gel showing DTT titrations of RT-PCR, with numbers corresponding to those in panel A. N is the RT-PCR negative control, and M is a 100 bp DNA ladder (New England Biolabs).

### 3.3.1.5. Elongation step

Generally 1 min of elongation is required per 2 kb of amplicon (Coyne *et al.*, 2004) and this was confirmed empirically. Using 1.5 min (as opposed to 1 min) extension period seemed to slightly increase the amount of RT-PCR product – lane 4 (1.5 min) has slightly more DNA than lane 3 (1 min) of Fig. 3–5. This slight increase in product when using 1.5 min extension time was reproduced twice. Therefore an extension time of 1.5 min was used.

**A**

| **Lane number** | ***T. pallidum* RNA** | **Elongation period at 72°C for 1 min or 1. 5 min** |
|---|---|---|
| **1** | $10^3$ | 1.0 |
| **2** | | 1.5 |
| **3** | $10^2$ | 1.0 |
| **4** | | 1.5 |
| **5** | $10^1$ | 1.0 |
| **6** | | 1.5 |
| **Negative control** | none | 1.5 |

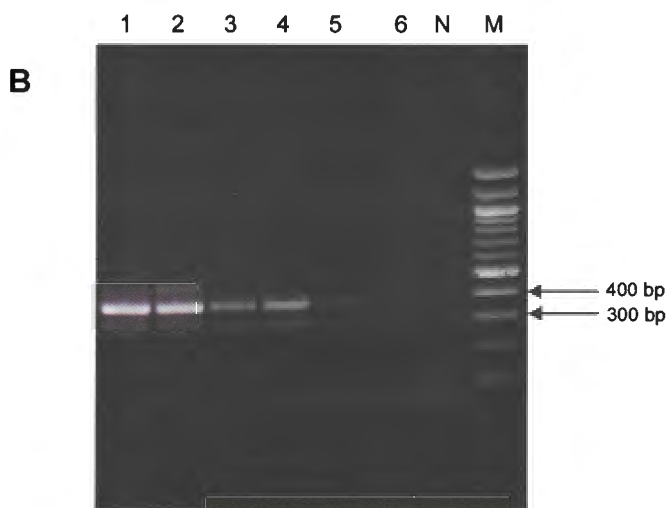



**Fig. 3–5. A.** The length of elongation time at 72°C and the RNA amount used for each sample. **B.** Agarose-EtBr gel with numbers corresponding to those in panel A, N is the RT-PCR negative control using water, and M is a 100 bp DNA ladder (New England Biolabs).

### 3.3.1.6. Heat denaturation of RNA prior to the RT-step

Heat denaturation can disrupt the secondary structure of RNA, which could possibly increase sensitivity (Kawasaki & Wang, 1989; Kuo *et al.*, 1997). However, it was empirically determined that if the RNA was not heat denatured, slightly more product was observed, in comparison to heat denaturing the RNA – lane 3 (no heat denaturation) has slightly more product than lane 4 (heat denaturation) of Fig. 3–6. This slight increase in product when performing no heat denaturation was observed three times. Since a heat denaturation step involves increased work and thus increased chance of contamination, it was decided not to heat denature the RNA in future RT-PCR reactions – especially given the lack of any benefit.

**A**

| **Lane number** | ***T. pallidum* RNA** | **Heat denaturation (94°C, 1 min) performed: Yes or No** |
|---|---|---|
| **1** | $10^3$ | No |
| **2** | $10^3$ | Yes |
| **3** | $10^2$ | No |
| **4** | $10^2$ | Yes |
| **5** | $10^1$ | No |
| **6** | $10^1$ | Yes |
| **Negative control** | none | Yes |

**B**

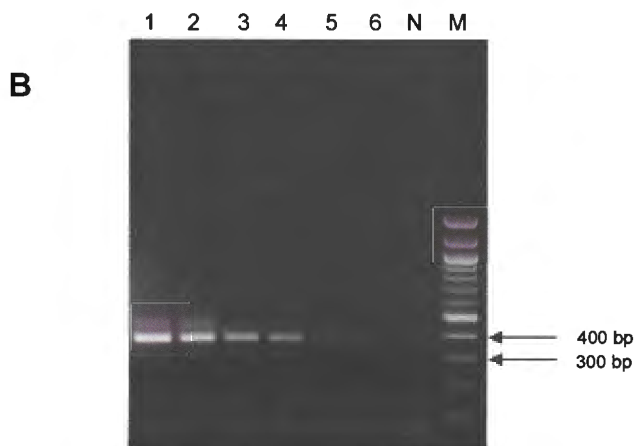



**Fig. 3–6. A.** The amounts of *T. pallidum* RNA used with or without heat denaturation of the RNA sample.
**B.** Agarose-EtBr gel depicting RT-PCR products when comparing heat denaturation of RNA prior to RT, vs. no heat denaturation. Numbers correspond to those in panel A, N is the negative control, M is a 100 bp DNA ladder (New England Biolabs).

#### 3.3.1.7. Other optimisations

Other optimisations, using increased amounts of M-MuLV Reverse Transcriptase RNase $H^-$ (Finnzymes) and DyNAzyme EXT DNA Polymerase (Finnzymes) as per 3.2.2.2 did not affect the amount of RT-PCR product formed. It was decided to continue using 2 U DNA polymerase and 10 U reverse transcriptase.

#### 3.3.1.8. Sensitivity of the RT-PCR assay for T. pallidum

Prior to optimisations, RT-PCR was performed on serial 10-fold dilutions of *T. pallidum* RNA, ranging from $10^5$ to $10^0$ organism equivalents, using baseline conditions described in section 3.2.2.1. RT-PCR product of 366 bp could be detected when using $10^3$ or more *T. pallidum* equivalents of RNA (Fig. 3–7, panel A). The product using $10^3$ *T. pallidum* RNA equivalents cannot be seen clearly on this gel, but when the gel was directly viewed, a faint band was visible.

Optimised RT-PCR conditions resulted in detection of $10^1$ *T. pallidum* equivalents of RNA (Fig. 3–7, panel B). These conditions, which were used for all subsequent RT-PCR reactions in this thesis, consisted of 1X RobusT Reaction Buffer (Finnzymes), 2 mM $MgCl_2$ (Finnzymes), 10 U M-MuLV Reverse Transcriptase RNase $H^-$ (Finnzymes), 2 U DyNAzyme EXT DNA Polymerase (Finnzymes), 0.4 mM each dNTP (Finnzymes), 50 U RiboLock RNase Inhibitor (Fermentas), and 0.5 pmol/µl each of sense and antisense primers (Department of Molecular and Cell Biology, UCT). The cycling conditions found to optimum were: 42°C (50 min); 94°C (2 min); 40 cycles of 94°C (30 s), 62°C (1 min) and 72°C (1.5 min); 72°C (5 min).

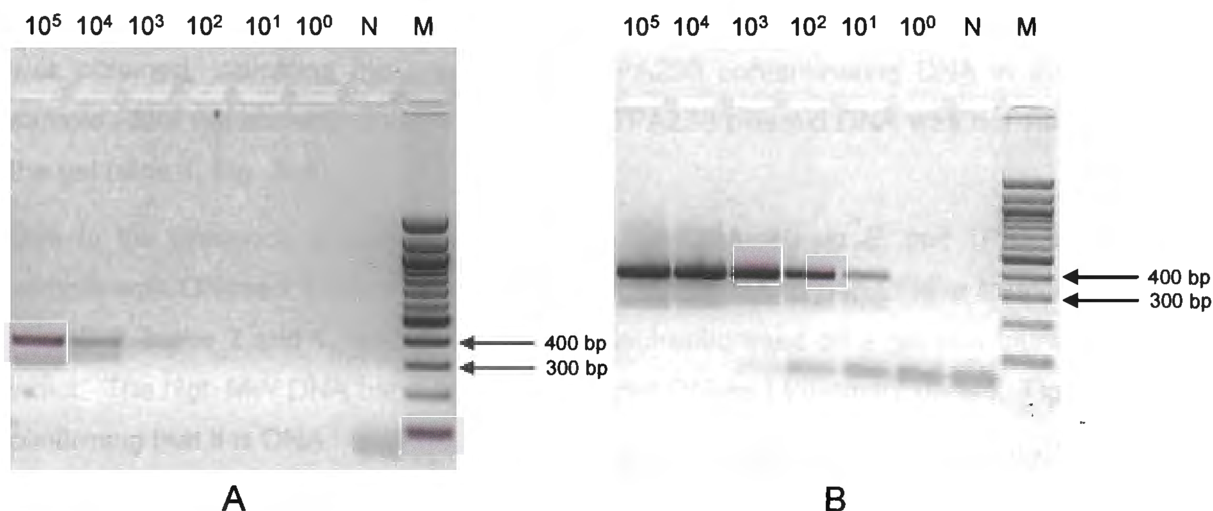



**Fig. 3–7. A.** Agarose-EtBr gel showing RT-PCR products when using varying *T. pallidum* RNA amounts, as indicated in the lanes, before optimisations were performed. N is the RT-PCR negative control using water, and M is a 100 bp DNA ladder (New England Biolabs).
**B.** Agarose-EtBr showing the sensitivity of RT-PCR after performing the optimisations, using RNA from the indicated amount of *T. pallidum* organisms, where N is the RT-PCR negative control and M is a 100 bp DNA ladder (New England Biolabs). These gel pictures are inverted so that the bands can be seen more clearly.

### 3.3.2. Evaluation of the positive control, *E. coli* TPA238

The RNeasy Mini Kit RNA extraction method recommends a maximum of $10^9$ cells per column, due to the column's maximum RNA binding capacity of 100 µg. Additionally if too many cells are used, incomplete lysis would occur with subsequently lower RNA yield and purity (Qiagen, 2001). Specific quantities of *E. coli* TPA238 were thus calculated by reading absorbances at 600 nm. It was determined that an absorbance of 2.1 related to $10^8$ cfu/ml of *E. coli* TPA238. RNA was extracted from $10^8$ *E. coli* TPA238.

To determine that the RNA was indeed extracted and that RNase contamination did not occur, RNA was electrophoresed on an agarose-TAE gel. *E. coli* TPA238 RNA was intact, as shown on an agarose gel (lane 1, Fig. 3–8). Two distinct, bright bands were visible, depicting the 23S and 16S rRNA bands. In addition, no smearing could be seen at the bottom of the gel which would be an indication of degraded RNA. Besides the two rRNA bands, a higher MW band was visible (lane 1, Fig. 3–8). After RNase treatment of the gel, the two rRNA bands disappeared, whereas the high MW band remained, indicating that the two bands were RNA and the higher band was DNA (data not shown).

PCR, employing the conditions described for the PCR stage of the RT-PCR assay (section 3.3.1.8), was performed, using the 90 pg and 275 pg of the extracted RNA

sample (90 pg equivalent to 1.7 x $10^3$ cells; 275 pg equivalent to 5 x $10^3$ cells). Product was obtained, indicating the presence of pTPA238 contaminating DNA in the RNA sample (data not shown). Interestingly, the pTPA238 plasmid DNA was not visible on the gel (lane 1, Fig. 3–8).

Due to the presence of contaminating pTPA238 DNA, 10 µg *E. coli* TPA238 RNA sample was DNase I treated (section 3.2.3.4). DNase and non-DNase treated RNA (Fig. 3–8, lanes 2 and 1, respectively) were electrophoresed on a gel and found to be intact. The high MW DNA band disappeared after DNase I treatment (lane 2, Fig. 3–8), confirming that it is DNA.

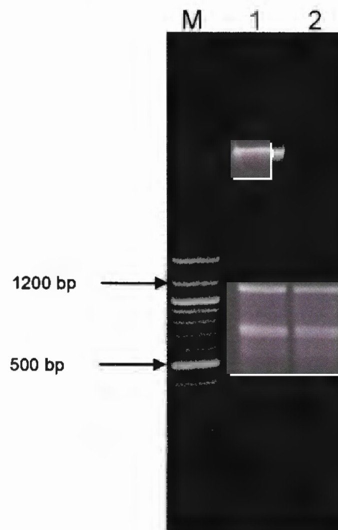



**Fig. 3–8.** Agarose-EtBr gel depicting *E. coli* TPA238 RNA before (lane 1) and after (lane 2) DNase I treatment. M depicts 100 bp DNA ladder (New England Biolabs).

PCR was performed on the DNase I-treated RNA sample (approximately 90 pg and 275 pg) to determine if any pTPA238 DNA remained (Fig. 3–9). A PCR positive control using 5 ng pTPA238 plasmid DNA as template was included to ensure that the PCR reaction was working. No PCR amplicons were observed when using the DNase I-treated RNA sample, indicating that there was no pTPA238 DNA contamination.

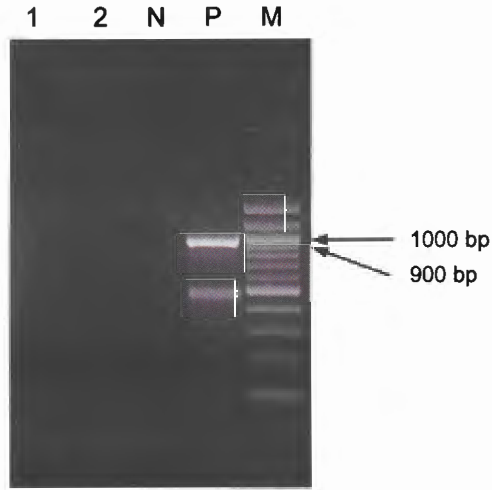



**Fig. 3–9.** Agarose-EtBr gel with PCR results using DNase I-treated *E. coli* TPA238 RNA, ~ 90 pg (lane 1) and ~ 275 pg (lane 2), N is the PCR negative control, P is the PCR positive control using plasmid pTPA238, and M is a 100 bp DNA ladder (New England Biolabs).

RT-PCR was subsequently performed on 90 pg and 275 pg of the DNase I-treated *E. coli* TPA238 RNA sample. A product of the expected size (~ 950 bp) was obtained (Fig. 3–10). This indicates that target RNA was transcribed from the *E. coli* TPA238 plasmid, pTPA238, and that it could be amplified by the RT-PCR assay. This in turn indicates that *E. coli* TPA238 could act as a reliable RNA extraction and reverse transcription control.

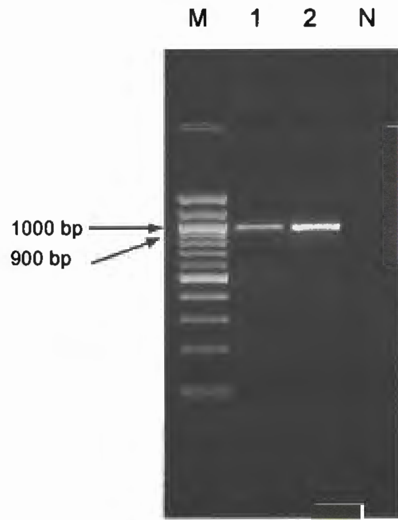



**Fig. 3–10.** Agarose-EtBr gel with RT-PCR products obtained from two different amounts of *E. coli* TPA238 RNA – approximately 90 pg (lane 1) and 275 pg (lane 2), N is the RT-PCR negative control and M is a 100 bp DNA ladder (New England Biolabs).

### 3.3.3. Optimisation of preparation and use of RNA from clinical samples

In order to ensure that RNA was extracted in an optimal way and used optimally in RT-PCR, various experiments were performed, such as determining what volume of extracted RNA was to be used in RT-PCR and how the positive control will be used. These optimisations were performed at least twice. If the results were not consistent, they were repeated a third or fourth time.

Experiments described in the following two sections (3.3.3.1 and 3.3.3.2) were performed in such a way as to mimic what would happen when working with clinical samples. *E. coli* TPA238 was spiked in 1.5 ml CSF, RNA was extracted using the RNeasy Mini Kit and eluted in 30 µl water.

#### *3.3.3.1. Determination of the volume of* E. coli *TPA238 extracted RNA, optimal for use in the RT-PCR assay*

The RNeasy Mini Kit allows RNA to be eluted from the RNeasy column in various volumes of water, however the minimum possible volume is 30 µl. Since the *T. pallidum* concentration in clinical samples is expected to be low, it would be best to use as much of the eluate as possible in the RT-PCR assay, and RNA was thus eluted in the minimum possible volume.

During preliminary investigations, RT-PCR products were not consistently obtained when using the entire 30 µl extracted RNA (e.g. extracted from $10^5$ *E. coli* TPA238). Conversely, when RNA extracted from $10^8$ *E. coli* TPA238 cells using the RNeasy Mini Kit, diluted to $10^5$ *E. coli* TPA238 equivalents, and used in RT-PCR, amplification products were obtained. This apparent inhibition, probably by high eluate volume, prompted the evaluation of the optimal fractions of extracted RNA to be used in the RT-PCR experiments.

Unfortunately *T. pallidum* cells were not available, therefore the volume of eluted RNA to be used in the RT-PCR assay was approximated by using *E. coli* TPA238 cells.

RNA was extracted from $10^4$ *E. coli* TPA238 suspended in 1.5 ml CSF, and eluted in 30 µl water. This was performed in triplicate, and the eluates were combined to obtain a pooled eluate volume of ~ 90 µl. Different volumes of the eluate (1, 3, 10, 15, 20, 25 µl) were used as templates in the RT-PCR reactions. As can be seen in lane 6, Fig. 3–11, inhibition occurred when using 25 µl eluate. As can be seen in lanes 3 – 5, Fig. 3–11, using 10, 15 or 20 µl of eluate appeared to yield a similar quantity of amplification product. In order to maintain a balance between potentially using too little

RNA (10 µl) and too high a volume of eluate (20 µl) with potential inhibition, 15 µl RNA would be used when evaluating the assay.

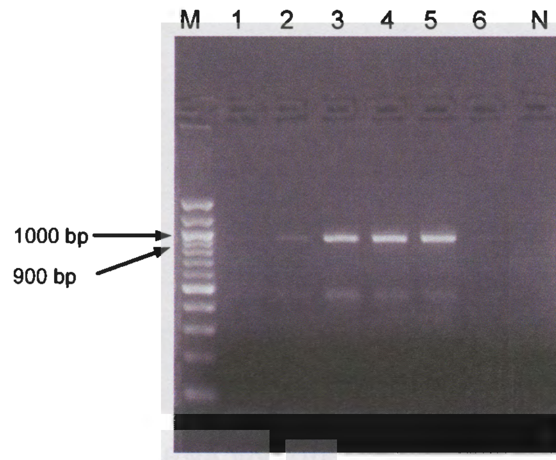



**Fig. 3–11.** Agarose-EtBr gel showing titrations of RNA volumes used in RT-PCR. Varying volumes of extracted RNA from $10^4$ *E. coli* TPA238 were used in the RT-PCR reactions: 1, 3, 10, 15, 20, 25 µl of 30 µl extracted RNA (lanes 1 – 6, respectively). Lane N is the negative RT-PCR control and M is a 100 bp DNA ladder (New England Biolabs).

### *3.3.3.2. Optimisation of the use of* E. coli *TPA238 positive control*

The first step of this section was to determine the amount of *E. coli* TPA238 that could consistently be detected by the assay. Following that, both *T. pallidum* and *E. coli* TPA238 RNA were used in the same reactions to determine if *E. coli* TPA238 has an effect on the sensitivity of the assay.

In order to determine the amount of *E. coli* TPA238 that should be added to the CSF samples to obtain a reliable result, RT-PCR was performed on RNA extracted from different quantities of the positive control. RNA was extracted from $10^4$, $10^3$, $10^2$, $10^1$ and $10^0$ *E. coli* TPA238 cells suspended in 1.5 ml CSF, and RT-PCR performed on 15 µl of the eluate. This was performed in duplicate. RNA from $10^4$ *E. coli* TPA238 can be detected (lanes 7 and 8, Fig. 3–12). Although RNA from $10^3$ *E. coli* TPA238 cells could be detected, this was not consistent (lanes 5 and 6, Fig. 3–12; and other data not shown); whereas RNA from $10^4$ *E. coli* TPA238 cells could be consistently detected.

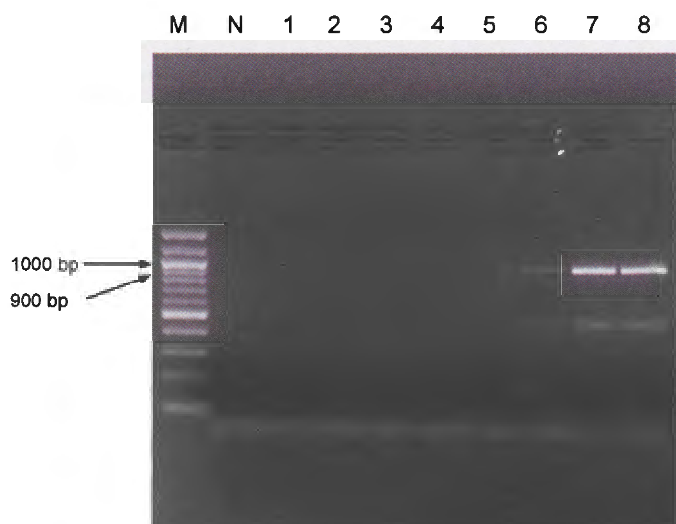



**Fig. 3–12.** RT-PCR products using RNA from $10^1$ (lanes 1 and 2), $10^2$ (lanes 3 and 4), $10^3$ (lanes 5 and 6) and $10^4$ *E. coli* TPA238 (lanes 7 and 8), M depicts a 100 bp DNA ladder (New England Biolabs) and N depicts the RT-PCR negative control.

To determine whether the presence of *E. coli* TPA238 would affect the sensitivity of the assay for *T. pallidum*, varying amounts of *T. pallidum* and *E. coli* TPA238 RNA were used in the same RT-PCR reactions (Fig. 3–13, panel A). This was performed for two reasons. Firstly, to determine whether the same sensitivity as determined previously when using *T. pallidum* on its own (section 3.3.1.8) can be reproduced, when using *T. pallidum* and *E. coli* TPA238 in the same reaction. Secondly, to determine the amount of *E. coli* TPA238 RNA to be used to obtain the same *T. pallidum* sensitivity (section 3.3.1.8), if the addition of *E. coli* TPA238 does decrease the sensitivity of the assay for *T. pallidum*. A maximum amount of RNA from $10^3$ *T. pallidum* organisms was used as it was thought that the amount of treponemal cells in the CSF, when evaluating the assay, would not be as high as $10^3$. This is acknowledged to be an assumption, since there is no available data relating to this issue.

In answer to the first question, it appears that *E. coli* TPA238 does slightly decrease the sensitivity of the assay for *T. pallidum* when using higher *E. coli* TPA238 amounts. As can be seen in lane 4, Fig. 3–13 ($10^4$ *E. coli* TPA238 and $10^3$ *T. pallidum*) there is slightly less *T. pallidum* product than in lane 13 ($10^3$ *T. pallidum* alone). Similarly, lane 7 ($7.5 \times 10^3$ *E. coli* TPA238 and $10^2$ *T. pallidum*) has slightly less product for *T. pallidum* than lane 14 ($10^2$ *T. pallidum* alone).

Secondly, using lower amounts of *E. coli* TPA238 did not render the assay less sensitive for *T. pallidum* – lane 5, Fig. 3–13 ($2.5 \times 10^3$ *E. coli* TPA238 and $10^2$ *T. pallidum*) and lane 6 ($5 \times 10^3$ *E. coli* TPA238 and $10^2$ *T. pallidum*) amplification of *T. pallidum* was equivalent to *T. pallidum* amplification when using *T. pallidum* on its

own (lane 14). It was decided not to use $2.5 \times 10^3$ *E. coli* TPA238 as higher amounts of *T. pallidum* ($10^3$) resulted in the near disappearance of the *E. coli* TPA238 product – lane 1 ($2.5 \times 10^3$ *E. coli* TPA238 and $10^3$ *T. pallidum*) yielded no *E. coli* TPA238 amplification product. The same or similar sensitivities were obtained when using *T. pallidum* alone compared to using *T. pallidum* and $5 \times 10^3$ *E. coli* TPA238 in the same reaction. Therefore $5 \times 10^3$ *E. coli* TPA238 cells will be used to spike clinical samples.

In this gel picture there are many non-specific bands present. The 500 bp band is the non-specific band due to *E. coli* TPA238 as discussed in Chapter 2 and the 1 100 bp band was occasionally observed when using *E. coli* TPA238 RNA (data not shown). The ~ 250 bp band is due to *T. pallidum* – this band can also be seen in other gel pictures e.g. Fig. 3–7, panel A, lane $10^5$. The origin of the 700 – 800 bp band that can be seen in lane 13 and very faintly in other lanes (e.g. lane 3), can also be seen in Fig. 4–1 when using *T. pallidum* RNA alone. The origin of the 700 bp band is not known. It is interesting that the ~ 700 bp band only appeared when combining *E. coli* TPA238 and *T. pallidum* RNA in the same reaction, and non-specific product formation appears more pronounced when combining *T. pallidum* and *E. coli* TPA238, which is slightly worrying. However, conditions were used that gave the best sensitivity for *T. pallidum*. The non-specific bands will be re-assessed when evaluating the assay on clinical samples.

**A**

| Lane number | *E. coli* TPA238 RNA | *T. pallidum* RNA |
|---|---|---|
| 1 | $2.5 \times 10^3$ | $10^3$ |
| 2 | $5 \times 10^3$ | |
| 3 | $7.5 \times 10^3$ | |
| 4 | $1 \times 10^4$ | |
| 5 | $2.5 \times 10^3$ | $10^2$ |
| 6 | $5 \times 10^3$ | |
| 7 | $7.5 \times 10^3$ | |
| 8 | $1 \times 10^4$ | |
| 9 | $2.5 \times 10^3$ | $10^1$ |
| 10 | $5 \times 10^3$ | |
| 11 | $7.5 \times 10^3$ | |
| 12 | $1 \times 10^4$ | |
| 13 | None | $10^3$ |
| 14 | | $10^2$ |
| 15 | | $10^1$ |

**B**

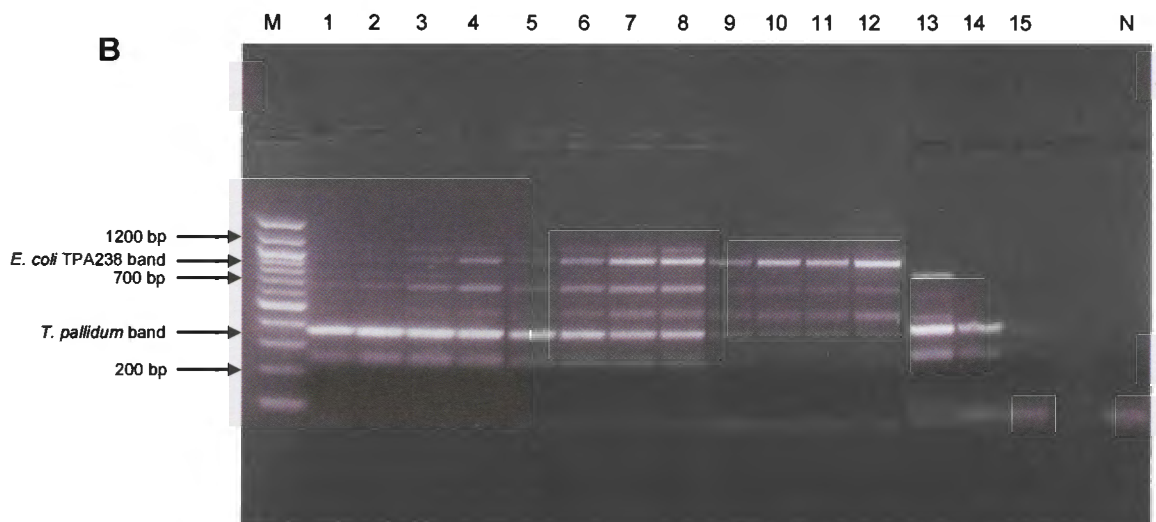



**Fig. 3–13. A.** The different amounts of *E. coli* TPA238 and *T. pallidum* RNA used, in RT-PCR reactions.
**B.** Agarose-EtBr gel showing the inter-relationship between *E. coli* TPA238 RNA and *T. pallidum* RNA to determine if *E. coli* decreases the sensitivity of the assay for *T. pallidum.* Numbers are as per panel A; M is a 100 bp DNA ladder (New England Biolabs) and N is the RT-PCR negative control.

### *3.3.3.3. RNA precipitations*

CSF samples that were collected for the evaluation of the assay were stored at –70°C. It is possible that, upon thawing, some of the *T. pallidum* cells might lyse and release their nucleic acids. For this reason, various RNA precipitation methods were tested (section 3.2.4.3), and RT-PCR was performed (section 3.3.1.8). Sodium acetate precipitation of RNA (section 3.2.4.3.i) did yield RT-PCR products (Fig. 3–14), but much RNA was lost in the precipitation process. Even more RNA was lost when precipitating RNA suspended in CSF compared to RNA suspended in saline (lanes C and S, Fig. 3–14). On repeating this method, results could not be reproduced. Ammonium acetate precipitation of RNA (section 3.2.4.3.v) was performed to test whether the method which was described by Centurion-Lara *et al.* (1997), presumably for nucleic acid precipitation after CSF and *T. pallidum* were frozen and then thawed, worked in this scenario. Unfortunately these RNA precipitation methods (sections 3.2.4.3.ii to 3.2.4.3.v) were unsuccessful, and no RNA precipitation will be used in preparation of RNA from clinical samples. The significance of this will be elaborated in the discussion, section 3.4.5.

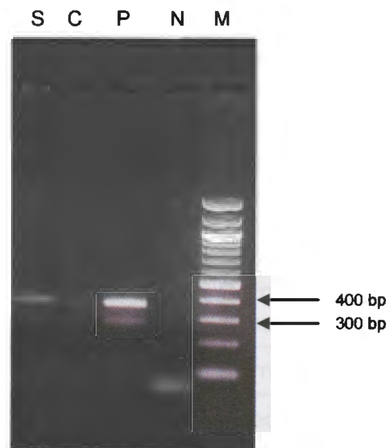



**Fig. 3–14.** Agarose-EtBr gel depicting the RT-PCR products obtained from *T. pallidum* RNA precipitated from saline (S) or CSF (C). P is the positive control using *T. pallidum* RNA – the same amount of RNA that was used in the S and C RT-PCR reactions if RNA precipitation were 100% efficient. N is the RT-PCR negative control and M is a 100 bp DNA ladder (New England Biolabs).

### 3.3.4. Specificity of the assay

In order to determine if the primers would amplify RNA from other bacterial species, RT-PCR (section 3.3.1.8) was performed on RNA extracted from a variety of bacteria (Appendix B.8). The bacterial species chosen (section 3.2.1.3) include causes of bacterial meningitis as well as laboratory contaminants, and provide a variety of Gram positive and Gram negative bacteria (Table 3–2). Due to the variation in yield of extracted RNA, a minimum of 22.5 ng RNA and a maximum of 100 ng RNA was used. Likewise, *T. pallidum* positive controls contained 22.5 ng and 100 ng RNA.

**Table 3–2.** Table showing bacteria from which RNA was extracted, and reasoning why they were chosen in the specificity of the assay.

| **Name** | **Clinical significance** |
| --- | --- |
| *Escherichia coli* | A cause of meningitis, mainly in neonates and very elderly. |
| *Staphylococcus epidermidis* * | Rare cause of infection, but common contaminant. |
| *Staphylococcus aureus* | Not a common cause of meningitis, but a common soft tissue pathogen. |
| *Enterococcus faecalis* | Uncommon cause of meningitis, rare laboratory contaminant. |
| *Pseudomonas aeruginosa* | Rare cause of meningitis, common clinical pathogen. |
| *Listeria monocytogenes* * | Uncommon cause of meningitis, but described especially in immuno-compromised patients. |
| *Streptococcus agalactiae* | Causes meningitis (mainly neonatal). |
| *Streptococcus pneumoniae* * | Common cause of meningitis and pneumonia. |
| *Neisseria meningitidis* * | Common cause of meningitis. |
| *Haemophilus influenzae* * | Used to be a common cause of meningitis, but less so now with vaccination. |
| *Mycobacterium tuberculosis* | The cause of tuberculous meningitis, which could be confused with neurosyphilis. |

* These are clinical isolates.

After performing the *T. pallidum* RT-PCR assay, amplification products were detected from seven organisms namely *Escherichia coli*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Neisseria meningitidis*, *Haemophilus influenzae* and *Mycobacterium tuberculosis* (Fig. 3–15, panels A and B). However the amplicons were not 366 bp in size (the size of the *T. pallidum* amplicon). Amplification of RNA from *M. tuberculosis* yielded amplification products very close or equal to 950 bp. This is a potential problem since this is also the expected size of the amplicon from

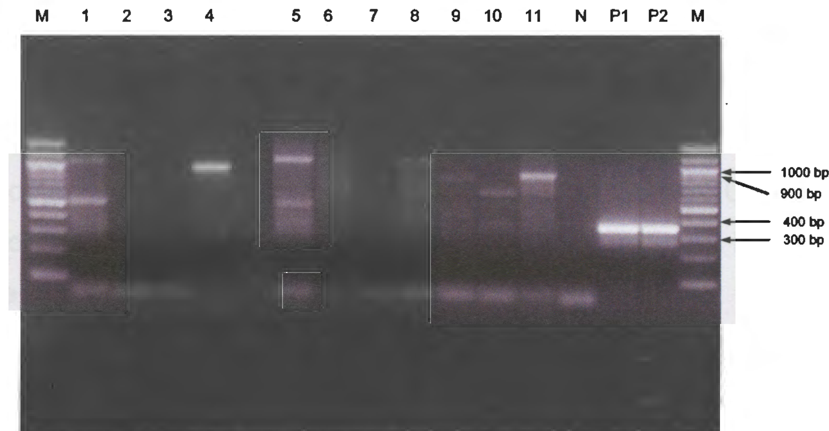
the positive control. Therefore, RT-PCR products from *M. tuberculosis* and *E. coli* TPA238 were electrophoresed together on an agarose gel and compared. This showed that the *M. tuberculosis* product migrated below the 950 bp positive control amplicon, at approximately 900 bp (data not shown). Therefore none of the non-specific products were 950 bp in size.

To further investigate the non-specific products, the RT-PCR assay was repeated on the seven RNA samples from which non-specific products were obtained. This time the positive control, *E. coli* TPA238, was added in the same RT-PCR reactions. Only the 950 bp *E. coli* TPA238 amplicon was obtained (Fig. 3–15, panels A and C). This indicates that *E. coli* TPA238 would most likely be preferentially amplified in the diagnostic RT-PCR assay, if any of the above organisms were present.

**A**

| Lane number | Organism | Lane number | Organism |
|---|---|---|---|
| 1 | *Escherichia coli* | 7 | *Streptococcus agalactiae* |
| 2 | *Staphylococcus epidermidis* | 8 | *Streptococcus pneumoniae* |
| 3 | *Staphylococcus aureus* | 9 | *Neisseria meningitidis* |
| 4 | *Enterococcus faecalis* | 10 | *Haemophilus influenzae* |
| 5 | *Pseudomonas aeruginosa* | 11 | *Mycobacterium tuberculosis* |
| 6 | *Listeria monocytogenes* | | |

**B**




**C**

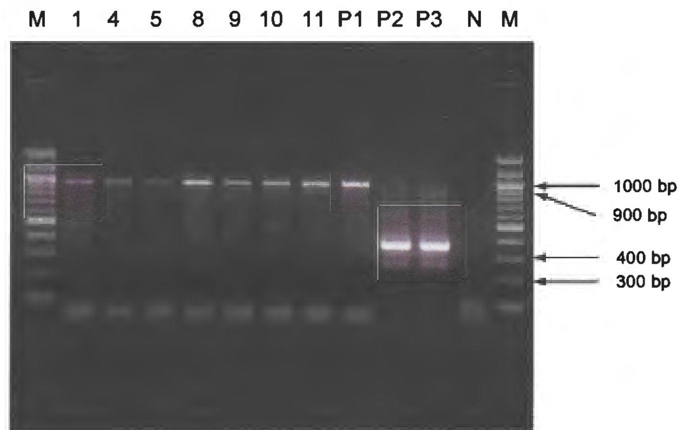



**Fig. 3–15. A.** Lane numbers corresponding to bacterial RNA used in the RT-PCR reactions.
**B.** Agarose-EtBr gel showing products formed when using *T. pallidum* sense and antisense primers on RNA extracted from various organisms (lanes 1 – 11, with numbers corresponding to organisms listed in panel A); N is the RT-PCR negative control; P1 is an RT-PCR positive control using 100 ng *T. pallidum* RNA and P2 is an RT-PCR positive control using 22.5 ng *T. pallidum* RNA. M is a 100 bp DNA ladder (New England Biolabs).
**C.** Agarose-EtBr gel with the RT-PCR products obtained when using RNA from bacteria 1, 4, 5, 8, 9, 10, 11 (as named in panel A) together with *E. coli* TPA238 ($5 \times 10^3$) RNA. P1, P2 and P3 represent RT-PCR products using RNA from $5 \times 10^3$ *E. coli* TPA238, 22.5 ng *T. pallidum* RNA and 100 ng *T. pallidum* RNA, respectively. N is the RT-PCR negative control and M is a 100 bp DNA ladder (New England Biolabs).

## 3.4. DISCUSSION

### 3.4.1. Optimisation of the RT-PCR reaction

Even though the RT-PCR assay will ultimately contain both *T. pallidum* and *E. coli* TPA238 RNA, *T. pallidum* RNA was used in the optimisations described (section 3.2.2.2), since detection of *T. pallidum* is the primary aim of the assay, and the quantity of *T. pallidum* in CSF may be limited (section 3.1). Prior to optimisation, $10^3$ *T. pallidum* RNA equivalents could be detected; this sensitivity was increased to detection of $10^1$ *T. pallidum* RNA equivalents, after optimisation (section 3.3.1.8).

During the optimisations, the $T_a$ was decreased from 64°C to 62°C to determine if the sensitivity could be increased. Annealing temperature can be defined as the temperature 5°C below the $T_m$ of the primer-template duplex, and the $T_m$ is the temperature at which half of the DNA duplex is dissociated (Hyndman & Mitsuhashi, 2003). The $T_m$ of the antisense primer is 64°C and that of the sense primer is 70°C. As mentioned in section 2.2.2, $T_a$ should theoretically be 59°C, but a $T_a$ of 62°C was successfully described (Centurion-Lara *et al.*, 1997). Seeing that $T_a$ was decreased from 64°C to 62°C, the $T_a$ is further away from $T_m$, thus primers are more likely to anneal to their target. Decreasing annealing temperature from 64°C to 62°C increased the amount of RT-PCR product obtained (section 3.3.1.1). Previously, due to problems with non-specific product formation, the annealing temperature was increased from 62°C to 64°C (Chapter 2, section 2.5.5.1). However, it was decided to use 62°C as sensitivity was increased, which is more important than slightly lower amounts of non-specific band, which would not interfere with interpretation of the assay.

It appeared that dNTPs, primers and $MgCl_2$ were limiting sensitivity for *T. pallidum* (sections 3.3.1.2 and 3.3.1.3), since increasing the dNTP, primer and $MgCl_2$ concentrations resulted in more RT-PCR products. It is important that the amount of dNTP and primers is sufficient otherwise sensitivity may be reduced (Grunenwald, 2003). In addition, $MgCl_2$ titrations had to be performed, as dNTPs and primers chelate $MgCl_2$, which DNA polymerase requires to function (Grunenwald, 2003). Higher concentrations of dNTPs can negatively affect product yield and specificity (Grunenwald, 2003) however when using 400 µM dNTPs and 2 mM $MgCl_2$, yield and specificity were not negatively affected. Increased primer concentration can result in lower specificity and low primer concentration can decrease sensitivity (Grunenwald, 2003). Increasing primer concentration did not adversely affect yield or specificity at 0.4 – 0.5 pmol/µl of primer.

DTT is usually required by RNase inhibitor to function (Sambrook *et al.*, 1989), but interestingly using no DTT seemed to increase sensitivity of the assay for *T. pallidum* (section 3.3.1.4). This could be because DTT cleaves disulphide bonds, which may denature proteins containing cystine residues (Stryer, 1995). DTT could have denatured reverse transcriptase or DNA polymerase if they contain cystine residues, thus rendering the assay less sensitive. It appears that the RiboLock RNase Inhibitor does not require additional DTT to function. A small amount of DTT is present in the RNase inhibitor, reverse transcriptase and DNA polymerase preparations, which results in a final concentration of ~ 0.24 mM DTT.

Heat denaturation was performed before the RT-step to disrupt any secondary structure of RNA. If any secondary structure (in the region where primers anneal) is stable at 42°C (RT-step temperature), primers would be unable to anneal or have more difficulty annealing to the template, and therefore product yield would be decreased or no product would be formed (Kawasaki & Wang, 1989; Kuo *et al.*, 1997; Tellier *et al.*, 2003). However, fewer or similar amounts of products were obtained when *T. pallidum* RNA was heat denatured prior to RT-PCR (section 3.3.1.6). Thus heat denaturation did not increase sensitivity suggesting that the target region probably does not form a secondary structure. Heat denaturing the RNA is more time consuming and could increase the chances of contamination as more pipetting is performed. In addition heat denaturation of RNA leaves the risk of not cooling down samples sufficiently before adding the heat-sensitive RT-PCR mix (specifically, reverse transcriptase and RNase inhibitor). Due to the disadvantages of heat denaturation and seeing that there was no increase in product yield, it was decided not to heat denature the RNA in future experiments.

### 3.4.2. RNA transcription from plasmid pTPA238

It was ascertained that the insert of plasmid pTPA238 was transcribed to RNA (section 3.3.2). Before performing RT-PCR, samples were DNase treated to ensure that no contaminating pTPA238 DNA remained in the RNA sample. If there was contaminating pTPA238 DNA, product could be formed during the PCR steps in the RT-PCR reaction, even if no RNA had been transcribed. However, because no contaminating DNA remained, this ensured that target RNA was transcribed and the RT-step is in fact working and that *E. coli* TPA238 could act as a reliable RNA extraction and RT control.

### 3.4.3. Determination of the volume of extracted *E. coli* TPA238 RNA, to be used in the RT-PCR assay

When using all or most of the RNA eluate (from $10^4$ *E. coli* TPA238) for RT-PCR, little or no RT-PCR products were obtained. It is not clear as to why high eluate volume inhibited RT-PCR, as RNA was precipitated and 'cleaned up' by buffers and centrifugation. RT-PCR can be inhibited by high amounts of RNA, but this would not be the case as the amount of RNA from $10^4$ *E. coli* TPA238 would be ~ 0.55 ng. Due to inhibition by high eluate volume, a titration using varying volumes of extracted RNA was performed, and it was decided to use 15 µl (of 30 µl extracted RNA).

The importance of this section is that *E. coli* TPA238 was used to estimate the optimal volume of eluate to be used when processing the CSF clinical samples, as *T. pallidum* cells were not available. RNA was extracted from *E. coli* TPA238 in the way that the clinical samples would be treated to better approximate the volume of eluted RNA to be used when working on clinical samples.

### 3.4.4. Amount of *E. coli* TPA238 with which to spike clinical samples

The amount of *E. coli* TPA238 cells with which to spike clinical samples, in order to obtain detectable positive control RT-PCR amplicons on an agarose gel, but not to decrease sensitivity of the assay for *T. pallidum*, was determined to be $5 \times 10^3$ cells. This decision was reached because when using $10^4$ or $7.5 \times 10^3$ *E. coli* TPA238, sensitivity for *T. pallidum* was decreased somewhat; and $2.5 \times 10^3$ *E. coli* TPA238 could not be detected when using higher concentrations of *T. pallidum* RNA. When using $5 \times 10^3$ *E. coli* TPA238 together with *T. pallidum* RNA, $10^1$ *T. pallidum* RNA could be detected – the same level as when no *E. coli* TPA238 was added.

Interestingly, the sensitivity of the RT-PCR assay for *T. pallidum* (on its own) was $10^1$, whereas only $2.5 \times 10^3$ *E. coli* TPA238 RNA could consistently be detected. The assay is thus 250 times more sensitive for *T. pallidum*. One reason could be that shorter amplicon generally amplify better than larger ones (Kleter *et al.*, 1998) (*T. pallidum* amplicon – 366 bp; *E. coli* TPA238 amplicon – 950 bp). A second reason could be because *T. pallidum* 16S rRNA is targeted, which makes up the bulk of total RNA samples. Messenger RNA makes up a smaller percentage of total RNA than that of rRNA and the *T. pallidum* primer binding sites in *E. coli* TPA238 are in mRNA, not rRNA.

The non-specific products observed when using both *T. pallidum* and *E. coli* TPA238 RNA in the same RT-PCR reactions may be a problem when performing the assay on clinical samples. This will be re-evaluated when the RT-PCR assay is performed on clinical samples.

### 3.4.5. RNA precipitations

The clinical CSF samples that would be evaluated were stored after routine laboratory tests were performed, and were not exclusively kept and handled for use in RT-PCR. They were stored at –70°C, and upon thawing cells might lyse releasing nucleic acids. Adding an RNA precipitation step to the sample processing may increase the total RNA yield from the sample, thus increase sensitivity. For this reason, RNA precipitation methods were performed to find the one that worked best.

Sodium acetate precipitation of RNA (section 3.2.4.3.i) did yield RT-PCR products in one instance. Much RNA was however lost in the RNA precipitation process, when compared to the positive control, even when using saline (Fig. 3–14). It is not known why so much RNA was lost during RNA precipitation of the saline solution. Even more RNA was lost when using CSF, possibly because CSF components were interfering with precipitation.

Unfortunately RNA precipitations using CSF (section 3.2.4.3.i) were not successful again, and none of the other RNA precipitation methods were successful. One possibility is that RNases may be present in the CSF. Adding RNase inhibitor to the CSF containing *T. pallidum* RNA would not have been practical because of expense. Alternatively, or additionally, protein could interfere with RNA precipitation. Interference of RNA precipitation by protein could be an important factor in the RT-PCR assay, as CSF protein concentrations can increase to over 40 mg/dl in patients with neurosyphilis (Larsen *et al.*, 1995). Using proteinase K, β-mercaptoethanol or performing $NH_4OAc$ precipitation of protein did not solve the problem. For this study, because RNA precipitation was unsuccessful, RNA will be extracted from clinical samples using only the RNeasy Mini Kit RNA extraction method.

It is possible that if lysis occurs, the assay would be less sensitive when working on clinical samples. Unfortunately, despite extensive investigations, RNA precipitation did not work. In future, when collecting CSF samples, the cells should be pelleted and stored in a reagent that stabilises and protects RNA (e.g. Qiagen RNAlater RNA Stabilization Reagent or RNAprotect Bacteria Reagent). The problem of lysis of cells would then be eliminated, and the assay would be expected to be more sensitive.

### 3.4.6. Specificity

When evaluating whether the sense and antisense primers would amplify RNA from other organisms, amplification products were obtained from seven of the 11 organisms tested, although the sizes did not correspond to *T. pallidum* or *E. coli* TPA238 amplicons. When performing these RT-PCR reactions, *E. coli* TPA238 was not added to the RT-PCR reactions. Adding *E. coli* TPA238 RNA would make formation of RT-PCR products from the other bacteria much less likely, thus not using *E. coli* TPA238 RNA was a more stringent test for specificity. However, when using bacterial RNA together with *E. coli* TPA238 RNA in the same RT-PCR reactions (Fig. 3–15, panel C), only the 950 bp amplicon from *E. coli* TPA238 was formed and no products from the other bacterial RNA were formed. This could suggest that these bacteria would not yield RT-PCR products in the *T. pallidum* diagnostic assay when spiking CSF with *E. coli* TPA238.

In hindsight, after having performed the evaluation, the specificity of the assay should have been tested on various amounts of RNA from each bacterial strain tested for specificity (section 3.3.4) as these bacteria could be present in varying concentrations in the sample. Performing this would also determine whether large numbers of non-specific organisms would be detected by the assay while competing with the positive control RNA (Fig. 3–15, panel C).

As there may be competition of primers between *T. pallidum* and the RNA from non-specific organisms (Fig. 3–15, panel B), this could decrease the sensitivity of the assay for *T. pallidum*. This is a potential limitation of the test that would need to be recognised – possibly to the extent that the assay should not be performed in cases of bacterial meningitis. Alternatively, this could be resolved by designing and evaluating a different set of primers – either still targeting the 16S rRNA gene, or else targeting a completely new target in the genome. In the latter case (and possibly also in the former), it would then be necessary to re-construct the positive control plasmid as well.

### 3.4.7. Summary and conclusions

In this section of the project, it was successfully determined that the insert of plasmid pTPA238 is transcribed to target RNA and yielded an RT-PCR product using the sense and antisense primers.

Optimisations increased the sensitivity of the assay from detection of $10^3$ to $10^1$ *T. pallidum* RNA equivalents.

Due to unsuccessful precipitation of RNA suspended in CSF, it was decided to use only the RNeasy Mini Kit RNA extraction method. In future work, the pelleted cells should be suspended in a stabilisation reagent.

The clinical CSF samples will be spiked with 5 x $10^3$ *E. coli* TPA238 bacteria and half of the 30 µl extracted RNA will be used.

None of the 11 bacteria tested for specificity of the assay yielded RT-PCR product(s) of 366 bp or 950 bp in size, which would indicate *T. pallidum* or the positive control, respectively.

# 4. CHAPTER FOUR – PRELIMINARY EVALUATION OF THE RT-PCR ASSAY ON CSF CLINICAL SAMPLES

## 4.1. INTRODUCTION

The final stage of the project was to conduct a small pilot study to evaluate the performance of this assay on CSF samples. It was conducted in conjunction with the Ophthalmology Department, Groote Schuur Hospital, Cape Town, South Africa.

Patients seen by the Ophthalmology Department with clinical features of uveitis or retinitis are often investigated for neurosyphilis, by means of serum RPR and FTA-ABS testing, as well as CSF investigations including syphilis serology (VDRL and FTA-ABS). The RT-PCR assay was performed on a selection of these CSF samples which had been stored at –70°C.

The results of the RT-PCR assays were then interpreted in combination with the clinical presentation, response to treatment and laboratory investigations. In addition, any technical problems in performing the assay on clinical samples were examined.

## 4.2. MATERIALS AND METHODS

### 4.2.1. Selection of patients and storage of CSF

The patients selected were those who presented to the Ophthalmology Department between November 2005 and March 2007, and were investigated for neurosyphilis at the discretion of the attending clinician. Request forms were highlighted by the clinician to alert the laboratory to store any residual CSF at –70°C as soon as possible after completion of routine laboratory investigations.

Routine diagnostic tests for neurosyphilis on CSF include FTA-ABS, VDRL, lymphocyte count, polymorphonuclear cell count and protein concentration determination; and serum FTA-ABS and RPR. These tests were performed in the National Health Laboratory Services diagnostic laboratory at Groote Schuur Hospital. Performing the RT-PCR assay on CSF samples that were left over after routine diagnoses were performed was approved by the UCT Research Ethics Committee (REC REF 338/2005) as was retrospective collection of clinical data from these patients. As no

extra specimens were taken from patients, and no extra clinical data was recorded, the need for informed consent was waived.

### 4.2.2. Performing the RT-PCR assay

The positive control, *E. coli* TPA238 was grown to a density of $10^8$ cells/ml as per section 3.2.3.1, and diluted to $10^7$ cells/ml in saline. It was suspended in 10 volumes of RNAlater RNA Stabilization Reagent (Qiagen), incubated overnight at 4°C and then stored at –20°C according to manufacturer's instructions (Qiagen, 2006).

The average volume of the CSF samples was ~ 500 µl and the entire volume was used for RNA extractions. Frozen CSF samples were thawed in a fridge. *E. coli* TPA238 cells (5 x $10^3$) were added to each CSF sample. A positive control consisted of 500 µl saline spiked with *E. coli* TPA238; and a negative control consisted of 500 µl saline only. Every batch on which the assay was performed contained a positive and negative control. RNA was extracted from CSF samples spiked with *E. coli* TPA238, the positive control and negative control, according to Appendix B.10, and RNA was eluted in 30 µl water. RT-PCR was performed as per section 3.3.1.8, using 15 µl of the RNA eluate. The RT-PCR products were electrophoresed on a 2% agarose-EtBr gel.

### 4.2.3. Clinical and laboratory data collection

Clinical data, including presenting symptoms, laboratory tests and other diagnostic test results, and response to treatment were collected by review of patients' folders. The data sheet used to record the information, can be found in Appendix E. The collection of data was only performed after the RT-PCR assay had been performed on all the clinical samples, and was performed by clinicians who were blinded to the RT-PCR results.

### 4.2.4. Categorisation of clinical and laboratory data

Currently, a diagnosis of neurosyphilis requires (Larsen *et al.*, 1995; Wicher *et al.*, 1999; Clyne & Jerrard, 2000):

- positive serum treponemal serology;
- CSF cell count of more than 5 mononuclear cells/$mm^3$ (i.e. lymphocytic cells);
- raised CSF protein levels of more than 40 mg/dl (or 0.4 g/l);
- positive CSF-FTA-ABS; and
- positive CSF-VDRL

Patients were classified on a rating of 1 – 4, as follows (section 1.8):

| Category | Laboratory results | Conclusion |
|---|---|---|
| 1 | Serum FTA-ABS negative, CSF-FTA-ABS negative | excludes syphilis |
| 2 | Serum FTA-ABS positive, CSF-FTA-ABS negative | excludes neurosyphilis |
| 3 | Serum FTA-ABS positive, CSF-FTA-ABS positive, CSF-VDRL negative | possible neurosyphilis |
| 4 | Serum FTA-ABS positive, CSF-FTA-ABS positive, CSF-VDRL positive | definite neurosyphilis |

## 4.3. RESULTS

### 4.3.1. RT-PCR assay results

The RT-PCR assay was performed in two batches of 15. After performing RT-PCR, the products were electrophoresed on an agarose gel (Fig. 4–1, panels A and B). Both these gel pictures have been inverted so that the faint bands can be seen more clearly.

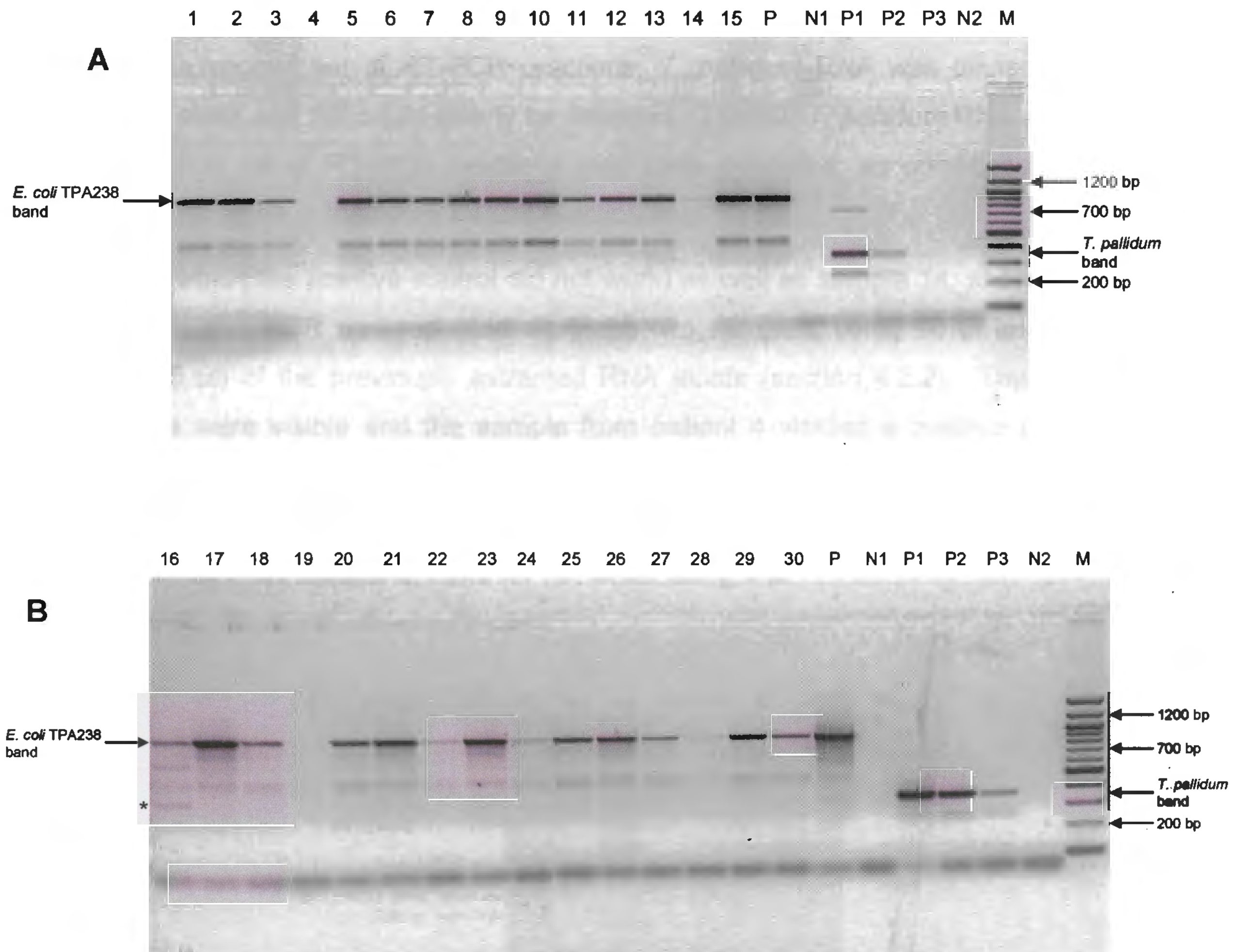



**Fig. 4–1. A.** Agarose-EtBr gel depicting RT-PCR amplicons derived from CSF samples 1 – 15 that were spiked with *E. coli* TPA238 (lanes 1 – 15). P is the RNA extraction positive control using only *E. coli* TPA238 and N1 is the extraction negative control using saline. N2 is the RT-PCR negative control. P1, P2 and P3 are RT-PCR positive controls using $10^3$, $10^2$, and $10^1$ *T. pallidum* RNA equivalents, respectively, and M is a 100 bp DNA ladder (New England Biolabs).

**B.** Agarose-EtBr gel showing RT-PCR amplicons derived from CSF clinical samples 16 – 30 (lanes 16 – 30), P is the RNA extraction positive control and N1 is the RNA extraction negative control. P1, P2 and P3 are RT-PCR amplicons derived from $10^3$, $10^2$, and $10^1$ *T. pallidum* RNA equivalents respectively; and N2 is the RT-PCR negative control. M is a 100 bp DNA ladder (New England Biolabs). The star indicates *T. pallidum* amplicons derived from CSF clinical sample 16.

The positive control, as evidenced by the presence of a 950 bp amplification product, worked in all but one of the samples – sample 4. The 950 bp positive control band was significantly fainter in samples 14, 19, 22, 24 and 28 suggesting a degree of inhibition of the assay. One CSF sample (number 16) yielded an amplicon of 366 bp indicating that *T. pallidum* was present in the sample, as well as the expected positive control amplicon of 950 bp.

In Fig. 4–1, panel A, $10^1$ *T. pallidum* could not be detected by RT-PCR. When performing the second set of RT-PCR reactions, *T. pallidum* RNA was diluted from concentrated stock and $10^1$ could clearly be detected. The $10^1$ *T. pallidum* RNA aliquot used for the first set of RT-PCR reactions may have degraded, as conditions used were exactly the same in the two RT-PCR reaction sets.

Sample 4 (in which the positive control did not work) as well as sample 14, showed no primer dimers. RT-PCR was repeated on these two samples, using 10 µl and 4 µl (instead of 15 µl) of the previously extracted RNA eluate (section 4.2.2). This time, primer dimers were visible and the sample from patient 4 yielded a positive control amplicon and *T. pallidum* amplicon when using 4 µl and 10 µl eluted RNA (Fig. 4–2). The sample from patient 14 again yielded the positive control amplicon when using 10 µl RNA in the RT-PCR reactions, however not when using 4 µl.

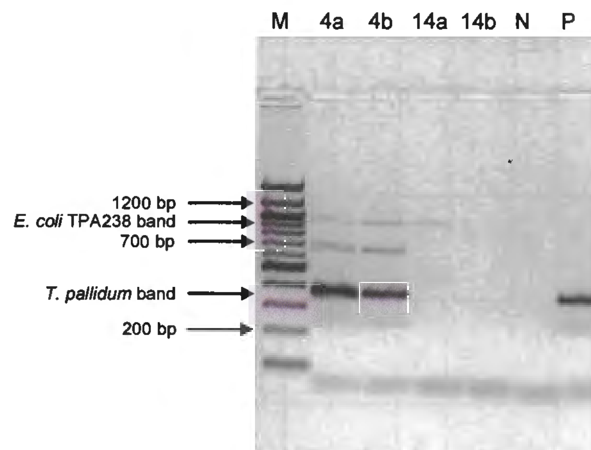



**Fig. 4–2.** Agarose-EtBr gel showing RT-PCR products using RNA extracted from samples 4 and 14. Lane 4a and 4b are the RT-PCR products when using 10 µl and 4 µl RNA, respectively; and lanes 14a and 14b are when using 10 µl and 4 µl RNA, respectively. N is the RT-PCR negative control and P is the RT-PCR positive control using $10^2$ *T. pallidum* equivalents of RNA.

## 4.3.2. Collection of clinical data

The clinical and laboratory data collected from the patients is summarised in Table 4–1. The results of categorisation are also shown in this table. Unfortunately, the folders for two of the patients were not available, and in some cases, laboratory investigations were not carried out as expected, resulting in some difficulties in categorising patients reliably. It should be noted that routine HIV testing was not carried out on most patients, thus HIV serostatus was not included in the analysis.

**Table 4–1.** Demographic information, diagnosis, laboratory results and treatment outcome of patients, compared to the RT-PCR assay result.

| # [a] | Age | Sex | Initial clinical diagnosis | Confirmed aetiology (if any) | Results of laboratory investigations | | | | | | | | | Treatment, follow up result (if available) | Category (1 – 4) [c] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | CSF [b] | | | | | | | serum | | | |
| | | | | | Poly* (/mm$^3$) | Lymph * (/mm$^3$) | Eryth* [d] (/mm$^3$) | Protein (g/l) | FTA-ABS | VDRL | RT-PCR | FTA-ABS | RPR [e] | | |
| 1 | 49 | F | papillitis | toxoplasmosis | 0 | 1 | 0 | 0.3 | – | – | – | + | – | IV penicillin*, co-trimoxale. Responded well to co-trimoxale. | 2 |
| 2 | 63 | F | optic atrophy | | 3 | 4 | +++ | ND* | – | – | – | + | - | IV penicillin. | 2 |
| 3 | 33 | M | syphilitic myelitis | | 0 | 36 | 0 | 0.75 | + | – | – | ND | + (256) | IV penicillin. | 3 [f] |
| 4 | 27 | M | papillitis | neurosyphilis | 75 | 680 | 0 | ND | + | + | + [g] | + | – | IV penicillin. Mild improvement. | 4 |
| 5 | 53 | F | stroke | | 0 | 11 | 0 | 0.48 | – | – | – | + | + (4) | | 2 |
| 6 | 31 | M | uveitis | | 0 | 11 | 0 | 0.27 | + | – | – | + | + (256) | IV penicillin. | 3 |
| 7 | 13 | F | endophthalmitis | | 0 | 5 | 0 | ND | – | – | – | – | + (16) | Evisceration. | 1 |
| 8 | 19 | F | uveitis | | 4 | 106 | 0 | 0.55 | + | – | – | + | + (64) | IV penicillin. | 3 |
| 9 | 38 | M | uveitis | | 0 | 0 | 0 | 0.29 | – | – | – | + | – | IM* penicillin (for secondary syphilis). | 2 |
| 10 | 55 | F | uveitis | | ND | ND | ND | ND | – | – | – | + | – | | 2 |
| 11 [h] | | | | | 0 | 17 | 0 | 0.19 | – | – | – | + | – | | 2 |
| 12 | 48 | F | optic neuritis | | 0 | 1 | 0 | 0.21 | – | – | – | + | + (1) | Steroids. Good response. | 2 |
| 13 | 38 | F | uveitis | | 1 | 61 | 0 | 0.72 | + | – | – | + | + | IV penicillin. Improved. | 3 |
| 14 | 26 | F | retinal vasculitis | | 0 | 7 | 0 | ND | – | – | – | + | + (64) | IV penicillin. | 2 |
| 15 | 63 | F | optic atrophy | | 0 | 1 | + | ND | – | – | – | + | – | IV penicillin. | 2 |

**Table 4–1 continued.**

| # [a] | Age | Sex | Initial clinical diagnosis | Confirmed aetiology (if any) | Results of laboratory investigations | | | | | | | | | Treatment, follow up result (if available) | Category (1 – 4) [c] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | CSF [b] | | | | | | | serum | | | |
| | | | | | Poly* (/mm³) | Lymph* (/mm³) | Eryth* [d] (/mm³) | Protein (g/l) | FTA-ABS | VDRL | RT-PCR | FTA-ABS | RPR [e] | | |
| 16 | 32 | M | posterior uveitis | | 10 | 255 | 0 | ND | + | – | + | + | + (256) | IV penicillin. Improved. | 3 |
| 17 | 42 | M | old corneal scar | | 0 | 0 | +++ | ND | – | – | – | + | – | Awaiting corneal graft. | 2 |
| 18 | 52 | M | keratitis, uveitis | | 0 | 2 | 0 | 0.55 | + | – | – | + | + | IV penicillin. | 3 |
| 19 [h] | | | | | 0 | 1 | 0 | 0.37 | – | – | – | ND | – | | 1 or 2 |
| 20 | 84 | F | posterior uveitis | | 0 | 1 | 0 | 0.41 | – | – | – | + | – | IV penicillin. Unchanged. | 2 |
| 21 | 51 | F | optic neuropathy | | 0 | 4 | 0 | ND | – | – | – | + | + (1) | IV penicillin. | 2 |
| 22 | 42 | M | uveitis | | 0 | 0 | 0 | 0.32 | – | – | – | + | + (1) | IV penicillin. | 2 |
| 23 | 25 | M | posterior uveitis | | 0 | 0 | + | 0.36 | + | – | – | | + (64) | IV penicillin. No new pathology. | 3 [f] |
| 24 | 35 | M | scleritis | | 0 | 9 | 0 | ND | – | – | – | + | – | IV penicillin, steroids. Responded well to steroids. | 2 |
| 25 | 43 | M | uveitis | | 0 | 1 | 0 | ND | – | – | – | + | + (1:4) | IV penicillin. Slow improvement. | 2 |
| 26 | 60 | F | panuveitis | sarcoidosis | 0 | 5 | 1 | ND | – | – | – | + | + (1:16) | Immunosupressants. | 2 |
| 27 | 37 | F | panuveitis | | 0 | 2 | + | 0.21 | – | – | – | + | – | Steroids, immunosuppressants. | 2 |
| 28 | 33 | M | panuveitis | toxoplasmosis | 1 | 16 | 0 | ND | – | – | – | – | – | Steroids. | 1 |

**Table 4–1 continued.**

| # [a] | Age | Sex | Initial clinical diagnosis | Confirmed aetiology (if any) | Results of laboratory investigations | | | | | | | | | Treatment, follow up result (if available) | Category (1 – 4) [c] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | CSF [b] | | | | | | | serum | | | |
| | | | | | Poly * (/mm$^3$) | Lymph * (/mm$^3$) | Eryth * [d] (/mm$^3$) | Protein (g/l) | FTA-ABS | VDRL | RT-PCR | FTA-ABS | RPR [e] | | |
| 29 | 34 | F | vasculitis, vitreous haemorrhage | | Unable to perform cell count because CSF was bloodstained | | ++++ | 1.35 | + | – | – | + | + (16) | IV penicillin. Unchanged. | 3 |
| 30 | 43 | M | neuroretinitis | | 5 | 0 | 88 | 0.36 | – | – | – | + | – | IV penicillin. Some improvement. | 2 |

* ND – not determined; Poly – polymorphonuclear cells; Lymph – lymphocytes; Eryth – erythrocytes; IV penicillin – intravenous penicillin G; IM – intramuscular.

[a] numbers correspond to those in Fig. 4–1.

[b] CSF clear and colourless unless otherwise noted. No culture results were positive for any of these CSF samples.

[c] The categories were determined as follows:

1 – Serum FTA-ABS negative, CSF-FTA-ABS negative : excludes syphilis

2 – Serum FTA-ABS positive, CSF-FTA-ABS negative : excludes neurosyphilis.

3 – Serum FTA-ABS positive, CSF-FTA-ABS positive, CSF-VDRL negative : possible neurosyphilis

4 – Serum FTA-ABS positive, CSF-FTA-ABS positive, CSF-VDRL positive : definite neurosyphilis

[d] During the time of CSF sample collection, the method of erythrocyte numeration differed. First, it was represented on a turbidity scale of 0 to ++++, where 0 is crystal clear and ++++ is opaque. The method then changed to determination of cell counts.

[e] The titre of the serum RPR is indicated in brackets.

[f] Serum FTA-ABS presumed to be positive on the basis of positive CSF-FTA-ABS.

[g] Positive result obtained on repeating RT-PCR (Fig. 4–2).

[h] The patient folders were not available, hence symptoms and treatment information is missing.

## 4.4. ANALYSIS OF RESULTS AND DISCUSSION

### 4.4.1. General comments

The clinical and laboratory data for analysis, including symptoms, laboratory investigation results, treatment and response to treatment information (if available) were compared to the RT-PCR results (Table 4–1). Since neurosyphilis is such a diagnostic problem and there is no convenient 'gold standard' to which the RT-PCR results can be compared, determining which patients have true neurosyphilis was made from a combination of these results. The RIT could be used as a 'gold standard' but is quite impractical – it is expensive and takes weeks to months obtain a diagnosis (Wicher *et al.*, 1999). Clinical presentation of patients was variable and included impaired visual acuity, pupillary abnormalities, pain, photophobia, headache, swollen discs, and dizziness. Some symptoms are compatible with neurosyphilis but none are specific for neurosyphilis (Singh & Romanowski, 1999). With this in mind, determining the performance of the RT-PCR assay compared to the current laboratory diagnosis of neurosyphilis, was a challenge.

Analysis is further complicated by issues such as bloodstaining as occurred with sample 29, for example. For this sample, cell counts could not be performed. To make matters more complex, if the CSF is bloodstained, this increases protein concentration, usually due to erythrocytes (Bonadio, 1992) which would result in an artificially high CSF protein concentration. The presence of blood in the CSF may also result in a false positive CSF-FTA-ABS result, since the CSF-FTA-ABS assay may be positive as a result of serum antibodies, not CSF antibodies (Davis & Sperry, 1979; Burke & Schaberg, 1985).

As can be seen in Table 4–1, polymorphonuclear and lymphocyte cell counts were also performed. If a disease is chronic, lymphocyte cell concentration is greater than polymorphonuclear concentration (Clinical and Laboratory Standards Institute, 2006). This is compatible with neurosyphilis, which is a chronic disease.

Table 4–2 depicts the number of RT-PCR positive results in comparison to the number of patients in categories 1 – 4.

**Table 4–2.** Depiction of the number of RT-PCR positive results in comparison to categories 1 – 4.

| Category | Number of samples within this category | Number of RT-PCR positive results | Comment about RT-PCR performance |
|---|---|---|---|
| Syphilis excluded (1) | 2 | 0 | True negative |
| Neurosyphilis excluded (2) | 19 | 0 | True negative |
| Neurosyphilis possible (3) | 8 | 1 (sample 16) | Difficult to interpret – see text for details. |
| Definite neurosyphilis (4) | 1 (sample 4) | 1 [a] | True positive |

[a] Positive result obtained on repeating RT-PCR (Fig. 4–2).

### 4.4.2. Positive results and significance in correlation with clinical information

Only one of 30 patients (patient 4) could be definitely diagnosed with neurosyphilis on the basis of the positive CSF-VDRL. CSF-VDRL is very specific for neurosyphilis (Burke & Schaberg, 1985; Hart, 1986). This patient had an elevated CSF lymphocyte count, positive CSF-FTA-ABS, positive CSF-VDRL and positive serum FTA-ABS, compatible with neurosyphilis. Symptoms were also compatible with neurosyphilis and the patient slightly improved after intravenous (IV) penicillin treatment. After performing the first RT-PCR reaction on this sample, no primer dimers were visible on the gel (Fig. 4–1, panel A, lane 4). It is strange that no primer dimers were visible, as the RT-PCR master mix (containing all the RT-PCR components, except for RNA) was made up, and aliquoted to each tube. RT-PCR was performed again for this reason, and less eluate was used as high eluate volume may have inhibited the initial RT-PCR reaction. On repeating the RT-PCR reactions, product was obtained for both *E. coli* TPA238 and *T. pallidum*, as would be expected (Fig. 4–2, lanes 4a and 4b). This result is taken as a true positive for the RT-PCR assay.

Inhibition of the positive control by high amounts of *T. pallidum* was observed for sample 4 (Fig. 4–2, lanes 4a and 4b). This is an unexpected problem as *T. pallidum* RNA concentrations were not expected to be at $10^3$ equivalents or greater; especially seeing that some cells might lyse. Lower concentrations of *T. pallidum* ($10^2$, $10^1$) did not inhibit the positive control (Fig. 3–13, lanes 6 and 10). The inhibition of the positive control by higher amounts of *T. pallidum* will have to be addressed.

Sample 16 depicts a patient with possible neurosyphilis (category 3) according to current diagnostic methods, and was positive for *T. pallidum* according to the RT-PCR assay. This was the only category 3 sample that was positive for *T. pallidum*. This

patient's lymphocyte count, positive CSF-FTA-ABS and positive serum FTA-ABS, and that the patient improved after IV penicillin treatment, are compatible with neurosyphilis. However, sample 16 yielded a very high serum RPR titre (256) which is consistent with secondary syphilis. The patient's CNS could have been invaded in early syphilis, which has been shown to occur in a study by Lukehart *et al.* (1988) (section 1.4); thus resulting in a positive RT-PCR. Clearly the RT-PCR assay will not be able to distinguish between secondary syphilis (if the CNS is invaded by *T. pallidum*) and neurosyphilis. As with any other laboratory test, clinical correlation is essential. Alternatively, shown by the negative CSF-VDRL, the patient could have had early neurosyphilis, without a CNS immune response yet.

Although the RT-PCR assay only detected *T. pallidum* RNA from two clinical samples (out of eight 'possible neurosyphilis' patients, and one 'definite neurosyphilis' patient), these results are encouraging as it indicates that the RT-PCR assay can work using CSF clinical samples.

### 4.4.3. Negative results and significance in correlation with clinical information and suggestions for future improvements

Twenty-one samples were categorised as 1 or 2 (excludes syphilis, or excludes neurosyphilis, respectively), and none of these 21 samples has a positive RT-PCR assay. These are thus true negative RT-PCR results.

With respect to category 3, it is very difficult to evaluate RT-PCR compared to a "possibly neurosyphilis" diagnosis, because, as has been mentioned, there is no convenient 'gold standard' for routine neurosyphilis diagnosis. The CSF-VDRL has a low sensitivity of 22 – 70% (Burke & Schaberg, 1985; Hart, 1986) and a negative CSF-VDRL result does not exclude neurosyphilis. Within category 3, there is a wide variety of results, with some more compatible with neurosyphilis (i.e. CSF having protein above 0.4 g/l and lymphocytes above 5/mm$^3$). However, category 3 was not subdivided into "more likely" and "less likely". For some samples, protein concentration was normal with lymphocyte concentration higher than 5/mm$^3$, and vice versa, and for other samples protein concentration was not determined. All of this made stratification within the group unreliable.

Having said the above, it is likely that category 3 may have patients without neurosyphilis for which the RT-PCR result was a true negative. However, it is also possible that the RT-PCR assay did not detect some category 3 samples containing *T. pallidum*. For example, samples 3, 8 and 13, all of which showed an RT-PCR

negative result, may all have come from patients with neurosyphilis as they all had raised lymphocyte counts and elevated protein. In addition, patient 13 showed clinical improvement after IV penicillin treatment. It must be remembered that this was a retrospective study and thus no firm criteria evaluating treatment response could be used, and no follow up CSF samples were taken. Compounding this, not all patients attended follow up, and response to treatment could not be evaluated at all.

If there were samples with *T. pallidum* that were missed by the assay, a likely reason is cell lysis, when thawing CSF. *E. coli* TPA238 cells were added after CSF was thawed and thus would not control for the freeze-thaw process. The assay was still being developed during collection of CSF samples. In future, the positive control cells will be added to the CSF, and the pellet stored in an RNA stabilisation reagent until RNA extraction is performed. This should have a double effect of increasing sensitivity of the assay for *T. pallidum* and the *E. coli* TPA238 could also control for storage conditions. In future it may be necessary to perform the RT-PCR reactions on a variety of eluate volumes for each patient (e.g. 5, 10 and 15 µl) in case there is RT-PCR inhibition. The inhibition would presumably affect *T. pallidum* detection more than *E. coli* TPA238 detection as *T. pallidum* numbers are usually low in CSF. When the *E. coli* TPA238 amplicon shows inhibition and there is no *T. pallidum* amplicon, the assay result should be treated with caution. This is because proteins and mononuclear cells may have interfered with RNA extraction and/or RT-PCR, and *T. pallidum*, which is usually present in low numbers in CSF, might then not be detected, even if *E. coli* TPA238 yielded a 950 bp amplicon.

### 4.4.4. Positive control and inhibition

The extent of inhibition of RNA extraction and RT-PCR for each sample (judged from the intensity of the *E. coli* TPA238 amplicons in comparison to the *E. coli* TPA238 positive control), as well as CSF chemistry and serological results are indicated in Table 4–3. As can be seen in Fig. 4–1, samples 14, 19, 22, 24 and 28 were dramatically inhibited. Samples 19 and 22 have no apparent reason for inhibition. Samples 14, 24 and 28 also have no apparent reason for inhibition, but perhaps protein concentration was high (these values were not determined).

Sample 16, which yielded an RT-PCR positive result for *T. pallidum,* had a relatively high number of lymphocytes (255/mm$^3$) and slight inhibition was observed. However, the RT-PCR assay yielded a positive result even though there were a relatively high number of lymphocytes.

The *E. coli* TPA238 positive control may be a reliable indicator of RT-PCR inhibition and whether RNA extraction was successful. It is difficult to say conclusively as for some samples, protein and/or cell concentrations were not determined.

Surprisingly, RT-PCR using sample 29 did not demonstrate significant inhibition, which would have been expected due to the high erythrocyte and protein concentrations. This suggests that other factors (other than cell and protein levels) may affect the sensitivity of the RT-PCR assay, and serves to highlight the previously discussed difficulties in predicting and eliminating inhibition.

**Table 4–3.** Comparison of laboratory tests performed on patients' CSF and inhibition of the RT-PCR assay.

| # | Results of laboratory investigations | | | | | | | Inhibition (0 – 2) [a] |
|---|---|---|---|---|---|---|---|---|
| | CSF | | | | | | | |
| | Poly * (/mm³) | Lymph * (/mm³) | Eryth * (/mm³) | Protein (g/l) | FTA-ABS | VDRL | RT-PCR | |
| 1 | 0 | 1 | 0 | 0.3 | – | – | – | 0 |
| 2 | 3 | 4 | +++ | ND* | – | – | – | 0 |
| 3 | 0 | 36 | 0 | 0.75 | + | – | – | 1 |
| 4 | 75 | 680 | 0 | ND | + | + | + [b] | 1 [b] |
| 5 | 0 | 11 | 0 | 0.48 | – | – | – | 0 |
| 6 | 0 | 11 | 0 | 0.27 | + | – | – | 0 |
| 7 | 0 | 5 | 0 | ND | – | – | – | 0 |
| 8 | 4 | 106 | 0 | 0.55 | + | – | – | 0 |
| 9 | 0 | 0 | 0 | 0.29 | – | – | – | 0 |
| 10 | ND | ND | ND | ND | – | – | – | 0 |
| 11 | 0 | 17 | 0 | 0.19 | – | – | – | 1 |
| 12 | 0 | 1 | 0 | 0.21 | – | – | – | 0 |
| 13 | 1 | 61 | 0 | 0.72 | + | – | – | 0 |
| 14 | 0 | 7 | 0 | ND | – | – | – | 1 |
| 15 | 0 | 1 | + | ND | – | – | – | 0 |
| 16 | 10 | 255 | 0 | ND | + | – | + | 1 |
| 17 | 0 | 0 | +++ | ND | – | – | – | 0 |
| 18 | 0 | 2 | 0 | 0.55 | + | – | – | 1 |
| 19 | 0 | 1 | 0 | 0.37 | – | – | – | 1 |
| 20 | 0 | 1 | 0 | 0.41 | – | – | – | 1 |
| 21 | 0 | 4 | 0 | ND | – | – | – | 0 |
| 22 | 0 | 0 | 0 | 0.32 | – | – | – | 1 |
| 23 | 0 | 0 | + | 0.36 | + | – | – | 0 |
| 24 | 0 | 9 | 0 | ND | – | – | – | 1 |
| 25 | 0 | 1 | 0 | ND | – | – | – | 0 |
| 26 | 0 | 5 | 1 | ND | – | – | – | 0 |
| 27 | 0 | 2 | + | 0.21 | – | – | – | 1 |
| 28 | 1 | 16 | 0 | ND | – | – | – | 1 |
| 29 | Unable to perform cell count because CSF was bloodstained | | ++++ | 1.35 | + | – | – | 1 |
| 30 | 5 | 0 | 88 | 0.36 | – | – | – | 1 |

* ND – not determined; Poly – polymorphonuclear cells; Lymph – lymphocytes; Eryth – erythrocytes.

[a] Inhibition in comparison to the *E. coli* TPA238 positive control, represented on a scale of 0 – 2, where 0 = no inhibition; 1 = partial inhibition and 2 = total inhibition.

[b] Result of repeated RT-PCR (Fig. 4–2).

### 4.4.5. Non-specific bands

Previously when using both *E. coli* TPA238 and *T. pallidum* RNA in the same reaction, various non-specific products were obtained (Fig. 3–13, section 3.3.3.2). This was to be re-assessed when the RT-PCR assay was evaluated. Interestingly, in Fig. 4–1, lane 16, non-specific bands are 500 bp and 700 bp in size, when both *E. coli* positive control and *T. pallidum* RNA were present in the same RT-PCR reaction. Whereas, Fig. 4–2, lanes 4a and 4b, contain 250 bp and 700 bp bands as non-specific bands. The non-specific bands are thus still visible when performing the assay on clinical samples, but do not appear to be as prominent as seen in the experiments discussed previously where *T. pallidum* and the positive control RNA were used in the RT-PCR reactions (Fig. 3–13), where there were up to seven bands. In figures 4-1 and 4-2 there are up to four bands. Although it would be ideal to eliminate these non-specific products entirely, they are not the size of the *T. pallidum* or positive control amplicons. The more important factor is sensitivity for *T. pallidum* and eliminating the non-specific bands would probably decrease sensitivity.

### 4.4.6. Summary and conclusions

This section of the project was a small pilot study, using CSF samples left over after routine diagnostic laboratory tests. The aim of this pilot study was to determine whether the assay would work in principle, and in particular, whether the use of the constructed positive control would function as envisaged.

Preliminary results suggest that the RT-PCR assay can be used on CSF samples. The RT-PCR assay detected *T. pallidum* from two CSF samples – one from a patient with definite neurosyphilis, and one from a patient with features suggestive of neurosyphilis or secondary syphilis. The RT-PCR result has to be read in conjunction with patient's symptoms as discussed in section 4.4.2. In order to improve the assay, it may be necessary in future to pellet cells and store them in an RNA stabilisation reagent, and to use 5, 10 and 15 µl eluted RNA in the RT-PCR reactions. These would potentially make the assay more reliable and sensitive. A possibility is to use nested primers and Noordhoek *et al.* (1991) found that this increased sensitivity and specificity for the PCR reactions. This would involve performing RT-PCR using a set of primers, then performing PCR on an aliquot of the PCR amplicons, using nested primers. Nested primers amplify a region within the original target region. The drawback to using nested primers would be the increased chance of contamination which goes hand in hand with the increased sensitivity (Apfalter *et al.*, 2002). In addition, primers could be

labelled with fluorescent dyes, and products could be detected using an ABI 310 Prism Genetic Analyzer. Liu *et al.* (2001) found that this increased the sensitivity of PCR. Inhibition of the positive control by higher amounts of *T. pallidum* must be investigated further.

The RT-PCR assay must still be evaluated more thoroughly. This would involve making sure that all specified laboratory investigations are carried out (as discussed earlier, in this study some laboratory information was not available), and that treatment and follow up information is obtained. In addition, the clinician can be given a standardised questionnaire to fill out for each patient. This would ensure that samples are analysed in the same way and could be correctly categorised. In addition, to evaluate the assay more thoroughly, negative control patients can be included – if it is known that the patients' symptoms are not compatible with syphilis or if laboratory investigations confirm that their symptoms are only due to another organism; as well as patients who have conditions as listed in Table 1–5. Also, possibly, the 'gold standard' of RIT could be used, in order to evaluate the performance of the RT-PCR assay more accurately. This would eliminate the problem of evaluating the assay against the 'possibly neurosyphilis' category. A wider range of patients would need to be evaluated, selected possibly from the Neurology Department.

# 5. CHAPTER FIVE – SUMMARY AND FUTURE WORK

Clinical diagnosis of neurosyphilis is difficult as it manifests non-specific symptoms which can be confused with other diseases. Laboratory diagnosis is also problematic. The CSF-FTA-ABS is very sensitive but has a low specificity for neurosyphilis. On the other hand, the CSF-VDRL has high specificity for neurosyphilis, but a low sensitivity. The purpose of the study was to develop a sensitive and specific diagnostic assay for neurosyphilis, which included a positive control for each sample.

CSF samples were spiked with bacteria containing a positive control plasmid. RNA was extracted and used for RT-PCR, following which the amplicons were electrophoresed on an agarose gel. The size of the *T. pallidum* amplicon is 366 bp and that of the positive control is 950 bp. The purpose of the positive control is to monitor RNA extraction and RT-PCR.

The positive control plasmid was constructed in two phases. In the first stage, a 366 bp region from the *T. pallidum* 16S rRNA gene was amplified by PCR using the sense and antisense primers. This was inserted in the pGEM-T Easy Vector, to form phase I plasmids. Performing PCR on these plasmids yielded amplicons of 366 bp. A phase I plasmid, pTPA108, was digested within the *T. pallidum* insert using the restriction enzyme *Bsa*BI, and was dephosphorylated. Spacer DNA, generated by digesting *E. coli* JM109 genomic DNA with RE *Eco*RV, was inserted into the *T. pallidum* 366 bp insert, to form phase II (positive control) plasmids. The addition of spacer DNA was performed in order to allow for size-based differentiation of RT-PCR amplicons originating from *T. pallidum* and that of the positive control. Performing PCR on phase II plasmids yielded an amplicon 2 – 3 times the size of the *T. pallidum* amplicon. The phase II plasmid, pTPA238 was used as the positive control plasmid.

It was shown that the insert of plasmid pTPA238 within *E. coli* TPA238, is transcribed to form RNA and yielded RT-PCR product of the correct size. Thus it should act as an RNA extraction and RT-PCR control.

The sensitivity of the RT-PCR reaction for *T. pallidum* was optimised by evaluating various parameters such as primer, $MgCl_2$, dNTP, DTT, DNA polymerase and reverse transcriptase concentrations, time of the elongation step and annealing temperature. The sensitivity of the assay was increased from $10^3$ *T. pallidum* equivalents to $10^1$ *T. pallidum* equivalents of RNA, as a result of these optimisations.

RNA was eluted in the minimum possible volume (30 µl) from the RNeasy Mini column in order to be able to use as much as possible in the RT-PCR reaction, as the

*T. pallidum* concentration and CSF volume was expected to be low. Use of 25 – 30 µl of the eluate inhibited the assay to a degree, and 15 µl of the eluate was thus used. The reason for this apparent inhibition of RT-PCR is not clear, as RNA was precipitated and wash steps were performed. However, the fact that the volume of eluate may inhibit the assay is an important limitation to remember.

The clinical samples were spiked with 5 x $10^3$ *E. coli* TPA238 cells. This amount was chosen so that product could be consistently detected on an agarose gel, but that the sensitivity of the assay for *T. pallidum* would not be decreased.

The RT-PCR reaction using the *T. pallidum* primers was tested on various bacterial species, including common causes of meningitis, pathogens and laboratory contaminants. RNA from seven of 11 of these organisms yielded RT-PCR amplicons but not at a size of 366 bp or 950 bp. When using *E. coli* TPA238 together with the RNA from the seven bacteria, *E. coli* TPA238 RNA was preferentially amplified. Due to potential competition between *T. pallidum* and RNA from non-specific bacteria, other primers should be tested.

The RT-PCR assay was evaluated by a small pilot study using 30 CSF samples collected by the Department of Ophthalmology, Groote Schuur Hospital. The RT-PCR assay results were compared to laboratory tests such as CSF protein concentration, lymphocyte concentration, erythrocyte concentration, CSF-FTA-ABS, CSF-VDRL, serum FTA-ABS and serum RPR; and response to treatment. Patients were divided into four categories (number of patients indicated in brackets): syphilis excluded (2), neurosyphilis excluded (19), possible neurosyphilis (8), and definite neurosyphilis (1) according to the current diagnosis of neurosyphilis.

Twenty-one samples gave negative results according to the RT-PCR assay and current diagnostic categorisation (categories 1 and 2) and these were thus true negative RT-PCR results.

The RT-PCR assay was positive for *T. pallidum* for two samples, one from the 'definite neurosyphilis' category and one from the 'possible neurosyphilis' category. The 'definite neurosyphilis' patient had features highly suggestive of neurosyphilis and it was concluded that the RT-PCR result was a true positive. The 'possible neurosyphilis' patient could have either had secondary syphilis or neurosyphilis. This is a potential problem of the RT-PCR assay, as early syphilis (if the CNS is invaded by *T. pallidum*) would not be differentiated from neurosyphilis; but as for all laboratory tests, clinical correlation should be performed.

It was difficult to evaluate the assay's performance when using samples from 'possible neurosyphilis' patients. Some of these samples may have contained *T. pallidum*, but were not detected by the RT-PCR assay, possibly due to cell lysis when thawing of CSF.

In future, pelleted cells will be stored in an RNA stabilisation reagent. It is also suggested that in future 5, 10 and 15 µl of RNA eluate be used in RT-PCR reactions in case there is inhibition of the RT-PCR assay. The effect of high amounts of *T. pallidum* on the amplification of the positive control needs to be further investigated. When performing further evaluation of the assay, all patients' samples should have the same set of laboratory tests performed and the attending clinician fill in a standardised questionnaire, as well as that follow up is performed. In addition, more patients should be analysed. This would ensure better standardisation, and analysis of results would be more reliable. Nested primer RT-PCR can be performed, which could increase sensitivity of the assay. Alternatively, fluorescent-labelled primers could be used and the amplicons analysed, which should increase sensitivity. In addition, hybridising the amplicons with a probe, complementary to a section of the amplicon, by means of a Southern blot or dot blot should also increase sensitivity. These are all interesting factors that still need to be researched.

There are potential benefits for patients if, after further evaluation, the assay proves to be sensitive and specific, including: more reliable diagnosis of neurosyphilis with earlier specific therapy; reduction in unnecessary investigations and empiric treatment; the assay, once validated, will improve the capacity of the diagnostic laboratory at Groote Schuur Hospital, and it is possible that the assay could be offered for specimens taken at other institutions as well.

Neurosyphilis and tuberculous meningitis; and syphilitic uveitis and tuberculous uveitis manifest similar symptoms thus there is a need to distinguish between infection by *T. pallidum* and *M. tuberculosis*. One could establish a similar assay, as in this project, for detecting *M. tuberculosis* 16S rRNA via RT-PCR. One could then perform multiplex RT-PCR, using two sets of primers, specific for tuberculosis and syphilis, thus distinguishing if patients are infected with *T. pallidum* or *M. tuberculosis*. Another exciting topic to investigate would be the performance of the RT-PCR assay using ocular fluids, to distinguish between tuberculous and syphilitic infection.

The two positive RT-PCR results show promise and suggest that the RT-PCR assay can be used on CSF samples in the diagnosis of neurosyphilis, after further evaluation is performed.

# APPENDIX A. SOLUTIONS AND MEDIA

**Ammonium acetate ($NH_4OAc$), 7.5 M**

Dissolve 289.05 g $NH_4OAc$ in ~ 400 ml distilled water ($dH_2O$). Make up to 500 ml with $dH_2O$. Autoclave.

**Ampicillin (100 mg/ml)**

Dissolve 500 mg in 5 ml sterile $dH_2O$. Filter sterilise, divide into aliquots and store at –20°C.

**5-brom-4-chloro-3-indolyl- ß-D-galactopyranoside (X-gal), 20 mg/ml**

Make up 40 mg/ml in dimethyl sulphoxide. After X-gal has dissolved, add an equal volume of $dH_2O$. Aliquot and store at –20°C.

**Calcium chloride ($CaCl_2$), 0.1 M**

Dissolve 14.7 g $CaCl_2 \cdot 2H_20$ in ~ 800 ml $dH_2O$. Make up to 1000 ml with $dH_20$ and autoclave.

**Cetyltrimethylammonium bromide (CTAB)/sodium chloride (NaCl)**

Dissolve 4.1 g NaCl in 80 ml $dH_2O$ and slowly add 10 g CTAB while heating and stirring. Adjust to 100 ml with $dH_2O$.

**Chloroform:isoamylalcohol**

Combine at a ratio of 24:1.

**Diethylpyrocarbonate (DEPC)-treated water**

Use 2 ml DEPC per 1 l $dH_2O$. Shake to dissolve DEPC. Stand for about 2 h, and then autoclave.

**Enzymatic lysis buffer (ELB)**

Final concentrations are 20 mM Tris-Cl, pH 8; 2 mM EDTA; 1.2% Triton X-100 and 20 mg/ml lysosyme. For 10 ml ELB, combine 40 µl of 0.5 M EDTA, 200 µl of 1 M Tris-Cl, pH 8 and 200 mg lysosyme. Make up to 10 ml with $dH_2O$. Aliquot and store at –20°C.

**Ethanol (EtOH), 70% w/v**

Add 70 ml absolute EtOH to 30 ml $dH_2O$.

**Ethidium bromide (EtBr), 10 mg/ml**

Dissolve 0.1 g EtBr in 10 ml sterile water. Store at 4°C and protect from light.

**Ethylenediaminetetraacetic acid (EDTA), 0.5 M**

Add 186.1 g $Na_2EDTA.2H_2O$ to 700 ml $dH_2O$ with stirring, and adjust to pH 8 by adding approximately 20 g NaOH. Adjust to 1 l with $dH_2O$ and autoclave.

**Glucose, 20% (w/v)**

Dissolve 20 g glucose in ~ 80 ml $dH_2O$. Adjust volume to 100 ml with $dH_2O$ and autoclave.

**Isopropyl-β-D-thiogalactopyranoside (IPTG), 200 mg/ml**

Dissolve 2 g IPTG in 8 ml $dH_2O$. Make up to 10 ml with $dH_2O$. Filter sterilise, aliquot and store at –20°C.

**Lysosyme (0.2 mg/ml)**

Dissolve 0.2 mg lysosyme in 1 ml sterile $dH_2O$. Divide in aliquots and store at –20°C.

**Magnesium chloride ($MgCl_2$), 0.1M**

Dissolve 20.3 g $MgCl_2.2H_2O$ in ~ 800 ml $dH_2O$. Adjust volume to 1 l with $dH_2O$ and autoclave.

**Proteinase K (20 mg/ml)**

Dissolve 200 mg proteinase K in 10 ml sterile $dH_2O$. Aliquot and store at –20°C.

**Ribonuclease (RNase) A, 10 mg/ml**

Dissolve 100 mg DNase-free RNase A in 10 ml of 10 mM Tris-Cl (pH 7.5) and 15 mM NaCl. Divide in aliquots and store at –20°C.

**Sample application buffer (SAB), 6X**

Dissolve 62.5 mg bromophenol blue and 10 g sucrose in 15 ml $dH_2O$. Add 1 ml 0.5 M EDTA and make up to 25 ml with $dH_2O$. Autoclave.

**Sodium acetate (NaAc ), 3 M, pH 5.2**

Dissolve 408.1 g sodium acetate.$3H_2O$ in 800 ml of $dH_2O$ and adjust pH to 5.2 with glacial acetic acid. Make volume up to 1 l with $dH_2O$ and autoclave.

**Sodium chloride (NaCl), 5 M**

Dissolve 292 g NaCl in ~ 700 ml $dH_2O$. Adjust to 1 l and autoclave.

**Sodium dodecyl sulphate (SDS), 10% w/v**

Dissolve 10 g SDS in 90 ml $dH_2O$. Heat to approximately 68°C to assist in dissolution. Adjust to 100 ml with $dH_2O$.

**Sodium hydroxide (NaOH), 10 M**

Dissolve 400 g NaOH in ~ 450 ml $dH_2O$. Add $dH_2O$ to 1 l and autoclave.

**Solution 1 (10X) (plasmid extraction)**

Combine 25 ml Tris-Cl pH 8 (1 M), 45.5 ml glucose (20% w/v) and 20 ml EDTA (0.5 M). Make to 100 ml with $dH_2O$. This solution contains 0.25 M Tris-Cl pH 8, 0.5 M glucose and 0.1 M EDTA. Dilute 1:10 before use.

**Solution 2 (plasmid extraction)**

Combine 2 ml 10 M NaOH and 10 ml SDS (10% w/v). Adjust to 100 ml with $dH_2O$. This solution should be made fresh weekly. This solution contains 1% SDS and 0.2 M NaOH.

**Solution 3 (plasmid extraction)**

Dissolve 147 g potassium acetate in approximately 250 ml $dH_2O$ and pH to 4.8 with glacial acetic acid. Make to 500 ml with $dH_2O$. This solution is 3 M with respect to potassium and 2 M with respect to acetate.

**Tris-acetate-EDTA (TAE) buffer, 50X**

Combine 242 g Tris, 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA (pH 8) in ~ 600 ml $dH_2O$ and dissolve. Make up to 1 l and autoclave. Dilute 1:50 to obtain 1X TAE buffer.

**Tris-Cl (1 M)**

Dissolve 12.1 g Tris in ~ 80 ml water. Add concentrated hydrochloric acid to the required pH, and make up to 100 ml with $dH_2O$. Autoclave.

**Tris-EDTA (TE) buffer**

Combine 1 ml Tris-Cl pH 8 (1 M) with 200 µl EDTA pH 8 (0.5 M) and make up to 100 ml with $dH_2O$. Autoclave.

**2X yeast-tryptone (2X YT) broth**

Add 16 g tryptone, 10 g yeast extract and 5 g NaCl to 900 ml $dH_2O$ and dissolve. Adjust to 1 l with $dH_2O$. Autoclave.

**2X YT agar**

As for 2X YT broth, except using 15 g agar / l.

# APPENDIX B. METHODS

## Appendix B.1

**Setting up and electrophoresing an agarose gel containing EtBr**

Adapted from Ausubel *et al.*, 1987.

1. Weigh the appropriate amount of agarose (Hispanagar) and add the appropriate volume of 1X TAE buffer, according to the percentage of agarose desired.
   Generally, a higher percentage of agarose is used when electrophoresing DNA of lower molecular weight on an agarose gel. For example, 2% (w/v) agarose was used for the 366 bp product, whereas 0.8 – 1% (w/v) agarose was used for genomic DNA or genomic DNA that had been digested with restriction enzymes.
2. Heat the mixture in a microwave oven until agarose has dissolved.
3. Allow agarose to cool to about 40°C, and add 10 mg/ml EtBr (Boehringer Mannheim), so that EtBr is at a final concentration of 0.5 µg per ml of agarose mixture.
4. Put heat resistant tape on the open sides of the gel container, insert comb and pour agarose to the required thickness.
5. Allow agarose to solidify until it becomes opaque.
6. Remove the comb and tape.
7. Put the agarose gel in a gel tank, filled with 1X TAE buffer to just cover the agarose gel.
8. Add the 6X SAB to the DNA samples, so that there is a final concentration of 1X SAB, and load the samples in the wells.
9. Set the voltage to the required amount (approximately 3.5 – 4 V/cm).
   An electric field is generated by voltage applied at the ends of an agarose gel. Due to the electric field, DNA molecules, which have negatively charged phosphates, electrophorese towards the anode. Larger DNA molecules migrate slower than smaller DNA molecules, and thus agarose gel electrophoresis allows size separation.

10. Allow the SAB to run half – three quarter of the length of the gel, depending on the size of the DNA.
    SAB consists of: bromophenol blue – to detect how far the DNA has electrophoresed; sucrose so that the DNA sinks into the well and does not float out; EDTA to inhibit any DNase enzymes that may be present as EDTA chelates $Mg^{2+}$ that is required for the enzyme to function.
11. Subject the agarose gel to UV (302 nm) irradiation using the Fotodyne Incorporated UV box, and capture using the Kodak 1D 3.6 program and Kodak EDAS 290 camera.

## Appendix B.2

**Quantification of DNA using λ genomic DNA standards**

Adapted from Coyne *et al.*, 2004.

1. Prepare lambda (λ) genomic DNA (Roche) at concentrations of 50, 100 and 200 ng/10 and load 10 µl onto an agarose gel containing 0.5 µg/ml EtBr.
2. Varying volumes of the DNA to be quantified are also loaded onto the gel. The gel is electrophoresed until the loading dye is 1 – 2 cm from the wells.
3. Subject the gel to UV (302 nm) irradiation in order to visualise the DNA.
4. The intensities of the standards are compared to the intensities of the DNA to be quantified, thus allowing an estimation of the DNA concentrations.

## Appendix B.3

**Preparation of competent *E. coli* JM109 cells by the $CaCl_2$ shock treatment.**

Adapted from Dagert and Ehrlich, 1979; Ausubel *et al.*, 1987; Sambrook *et al.*, 1989; Coyne *et al.*, 2004.

1. Inoculate a loopful of *E. coli* JM109 into 5 ml 2X YT broth and incubate at 37°C with shaking, overnight.
2. Dilute this overnight culture 1:200 into 500 ml 2X YT broth in a 2 l flask.
3. Grow the *E. coli* culture to early log phase (absorbance at 600 nm = 0.3 – 0.6).
4. Collect cells by centrifugation at 5000 rpm for 5 min, at 4°C.
5. Keep the cells ice cold in all remaining steps.
6. Resuspend the cells in 1 culture volume ice cold 0.1 M $MgCl_2$. Leave on ice for 1 min.

7. Collect the cells by centrifugation at 5 000 rpm for 5 min, at 4°C and resuspend in 0.5 culture volume of 0.1 M $CaCl_2$. Keep on ice for 1.5 h. Exposure of cells to $Ca^{2+}$ ions enables the cells to take up DNA.
8. Collect cells by centrifugation at 5 000 rpm for 5 min, at 4°C and gently resuspend in 0.1 culture volume 0.1 M $CaCl_2$.
9. Store the cells in the presence of approximately 15% (v/v) glycerol, at –70°C.

## Appendix B.4

**Transformation of competent *E. coli* JM109 cells.**

Adapted from Dagert and Ehrlich (1979), Ausubel *et al.*, 1987; Sambrook *et al.*, 1989; Promega, 2003 and Coyne *et al.*, 2004.

1. Centrifuge ligation mix briefly and add part or all of the ligation mix to Eppendorfs.
2. Thaw competent *E. coli* JM109 cells on ice, gently flicking them occasionally.
3. Transfer 100 µl cells to the Eppendorfs containing ligation mix, gently flick, and keep on ice for 20 min.
4. Heat shock at 42°C for 2 min. This is performed so that DNA can efficiently enter the cells.
5. Place tubes on ice for 2 min. This step was found to increase the transformation efficiency.
6. Add 900 µl 2X YT broth to each Eppendorf and incubate with shaking at 37°C for 1 h.
7. Plate the appropriate amount of cells on the appropriate media and incubate overnight at 37°C.

## Appendix B.5

**Extraction of plasmids by alkaline lysis**

Adapted from Birnboim and Doly, 1979; Ish-Horowicz and Burke, 1981; and Coyne *et al.*, 2004.

1. Inoculate 5 ml 2X YT broth supplemented with 100 µg/ml ampicillin with a loopful of bacteria. Incubate overnight at 37°C with shaking.
2. Centrifuge 2 ml culture in a 2 ml Eppendorf for 1 min at 14 000 rpm to pellet cells. Discard the SNF. Add another 2 ml of culture and collect cells as before.

3. Resuspend cell pellet in 200 µl solution 1 (containing Tris-Cl pH 8, glucose and EDTA).
   EDTA chelates $Mg^{2+}$ which is required by DNase to function, and glucose is used for pH control.
4. Incubate at room temperature for 10 min.
5. Add 400 µl solution 2 (containing SDS and NaOH) and mix well.
   This mixture is for cell lysis. In addition, NaOH denatures chromosomal DNA.
6. Incubate on ice for 10 min.
7. Add 300 µl pre-cooled solution 3, pH 4.8 (containing potassium acetate and glacial acetic acid).
   This is for neutralisation, and the chromosomal DNA forms an insoluble network.
8. Shake well and incubate on ice for 10 min.
   A white flocculent forms, depicting cellular protein and membrane precipitation.
9. Centrifuge for 10 min at 14 000 rpm.
10. Transfer the SNF (900 µl) containing plasmid DNA, to a fresh Eppendorf. Add 600 µl isopropanol, for precipitation, mix, incubate for 2 min at room temperature, then centrifuge at 14 000 rpm for 10 min to pellet plasmid DNA. Discard SNF.
11. Add 500 µl 70% ethanol (v/v) to the pellet, rinse by inversion, pour off ethanol and dab dry. Centrifuge briefly and remove remaining 70% ethanol by pipette.
12. Air-dry for 5 – 10 min, and resuspend pellet (mostly plasmid DNA) in 20 – 50 µl TE buffer, pH 8 and 8 µg DNase free RNase A. Incubate at room temperature for 30 min.

## Appendix B.6

**Large scale preparation of bacterial genomic DNA**

Adapted from Ausubel *et al.*, 1987.

1. Inoculate 100 ml 2X YT broth with the desired bacteria and incubate overnight at 37°C, with shaking
2. Centrifuge at 6 000 rpm for 10 min to pellet bacteria. Discard the SNF.
3. For Gram positive bacteria perform this additional step – incubate the cell pellet in ELB (20 mM Tris-Cl pH 8, 2 mM EDTA, 1.2% Triton X-100, 20 mg/ml lysosyme) and leave at 37°C overnight (Schnaitman, 1971; Levine *et al.*, 2002).

This additional step was added to aid in lysis of the Gram positive bacteria that possess thicker cell walls compared to Gram negative bacteria.

4. Resuspend the pellet, or mixture when using Gram positive bacteria, in 9.5 ml TE buffer pH 8, 0.5 ml 10% SDS, and 50 µl of 20 mg/ml proteinase K. Mix and incubate for 1 h at 37°C.
   SDS is added for lysis of bacteria and proteinase K for digestion of proteins.
5. Add 1.8 ml 5 M NaCl and mix thoroughly.
6. Add 1.5 ml CTAB/NaCl solution and mix. Incubate for 20 min at 65°C.
7. Add 1 volume of 24:1 chloroform:isoamyl alcohol, mix and centrifuge at 7 000 rpm for 10 min at room temperature, to separate the phases. A white interface should be visible.
8. Transfer the aqueous SNF (containing nucleic acids) to a fresh tube.
   The aim of steps 4 – 7 is to remove protein, polysaccharides and cell wall debris. CTAB forms a complex with the latter forming a white precipitate, and then they are removed. Addition of NaCl is very important, as the nucleic acids remain in solution if the NaCl concentration is above 0.5 M. Should the salt concentration fall below 0.5 M NaCl the nucleic acid would precipitate.
9. Precipitate DNA with 0.6 volume of isopropanol, and mix gently until a stringy white DNA pellet precipitates out of the solution and condenses into a tight mass. Centrifuge at 15 000 rpm for 15 min.
10. Carefully discard the SNF. Wash the DNA pellet with 1 ml 70% ethanol and centrifuge for 5 min at 9 000 rpm.
11. Remove the 70% ethanol SNF and air-dry for approximately 10 min.
12. Resuspend the DNA pellet in 400 µl TE pH 8, containing 160 µg RNase A. Incubate for 30 min at room temperature. Store at 4°C overnight.

## Appendix B.7

### Ammonium acetate precipitation of DNA

Adapted from Coyne *et al.*, 2004.

1. Add 0.5 volume of 7.5 M $NH_4OAc$ to the DNA solution, and 2.5 volumes of 100% ethanol, for DNA precipitation, and mix.
2. Incubate at room temperature overnight.
3. Centrifuge at 14 000 rpm for 30 min at room temperature to pellet DNA.
4. Wash pellet with 1 ml 70% ethanol. Remove the SNF.
5. Allow the pellet to air-dry and resuspend in the required volume of buffer.

## Appendix B.8

**Qiagen RNeasy Mini Kit RNA extraction**

Qiagen, 2001.

1. Centrifuge the desired amount of culture at 14 000 rpm, 4°C for 5 min to pellet the cells, and remove SNF.
2. If cells were suspended in CSF, the pellet is washed by resuspending in 1 – 1.5 ml saline and centrifuging at 14 000 rpm, 4°C for 5 min. The SNF was removed.
3. Resuspend the cell pellet in TE, pH 8, with 0.4 mg/ml lysosyme (Bio Basic Inc.) for Gram negative bacteria, or 3 mg/ml for Gram positive bacteria. Incubate for 5 min at 25°C.
   Lysosyme is added to aid in digestion of the cell wall.
4. Add 350 µl lysis buffer RLT with ~ 10% β-mercaptoethanol and vortex. Buffer RLT contains guanidine thiocyanate.
   β-mercaptoethanol is added to act as an RNase inhibitor, breaking down disulphide bonds of RNase, in conjunction with guanidine thiocyanate.
   Guanidine thiocyanate destroys the three dimensional structure of proteins and dehydrates nucleic acids so that they bind to the silica column. Disruption of cell walls, plasma membranes and organelles of bacteria is accomplished by enzymatic lysosyme digestion and lysis buffer RLT (steps 3 and 4).
   Homogenisation, i.e. decreasing the viscosity of cell lysates (shearing high MW genomic DNA and other high MW cell components) is achieved by vortexing (step 4).
5. Add 250 µl absolute ethanol (Sigma-Aldrich) and pipette to mix.
6. Apply the sample to the RNeasy column within a collection tube, and centrifuge at room temperature for 15 s at 14 000 rpm. Discard flow-through.
7. Add 700 µl buffer RW1 to the column. Centrifuge for 15 s at 14 000 rpm at room temperature. Discard flow-through and collection tube.
8. Transfer the column to a new collection tube and add 500 µl buffer RPE to the column. Centrifuge for 15 s at 14 000 rpm at room temperature. Discard flow-through.
   Add 500 µl buffer RPE to the column, and centrifuge for 2 min at 14 000 rpm at room temperature. Buffers, RW1 and RPE, and centrifugation aid in removal of contaminants.
   Buffer RW1 contains salts, aiding in dehydration of nucleic acids and therefore

binding to the silica column; and breaking down the three dimensional structure of proteins. Buffer RPE contains low salt concentration and high alcohol percentage so that salts are washed off the column, but nucleic acids still remain on the column.

9. Place the column into a fresh 2 ml tube and centrifuge at 14 000 rpm, 1 min at room temperature, to remove any traces of ethanol.
10. Transfer the column to a 1.5 ml tube and elute using 30 µl of RNase-free water into the column, by centrifuging at 14 000 rpm for 1 min.
11. Put the same water (30 µl) through the column (step 11) and centrifuge at 14 000 rpm for 1 min.

## Appendix B.9

### RNase precautions when working with RNA

1. When working with RNA, filter tips, dedicated RNA pipettes and disposable gloves were used.
2. Glassware was baked at 240°C for 4 h.
3. Solutions were incubated with DEPC (Sigma-Aldrich) (0.2% v/v) for at least 2 h and then autoclaved.
   Autoclaving removes the remaining DEPC, which is important because DEPC is a suspected carcinogen and it carboxymethylates purine residues of RNA (Sambrook *et al.*, 1989).
4. For solutions that are not compatible with DEPC, e.g. Tris-containing solutions, more precautions were taken. Baked aluminium weighing boats and baked spoons were used to weigh ingredients, and the solution was prepared using DEPC-treated water.
   Tris is not compatible with DEPC, as they react which then inactivates DEPC (Ausubel *et al.*, 1989).
5. For an agarose RNA gel, agarose was weighed using a baked spoon and baked aluminium weighing boats, and baked glassware was used. In addition, TAE that had been specially prepared for RNA work was used and filter tips were used. RNA was added to 6XSAB, which had been prepared for RNA work.

## Appendix B.10

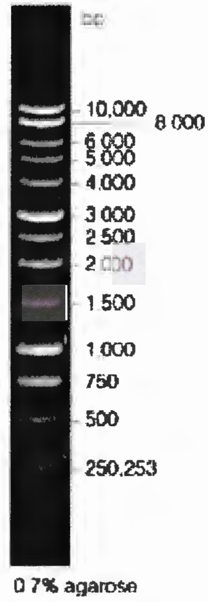
**Qiagen RNeasy Mini Kit RNA extraction, with on-column DNase digestion**

Qiagen, 2001.

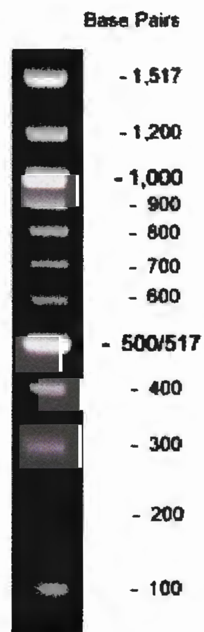
1. Centrifuge the sample at 14 000 rpm, 4°C for 5 min to pellet the cells, and remove SNF.
2. Wash the pellet by resuspending in 1 – 1.5 ml saline and centrifuging at 14 000 rpm, 4°C for 5 min. Cells were washed with saline in an attempt to remove any remaining inhibitors. The SNF is discarded.
3. Resuspend the cell pellet in TE, pH 8, with 0.4 mg/ml lysosyme (Bio Basic Inc.) for Gram negative bacteria. Incubate for 5 min at 25°C.
4. Add 350 µl buffer RLT with ~ 10% β-mercaptoethanol and vortex.
5. Add 250 µl absolute ethanol (Sigma-Aldrich) and pipette to mix.
6. Apply the sample to the RNeasy column within a collection tube, and centrifuge at room temperature for 15 s at 14 000 rpm. Discard flow-through.
7. Add 350 µl buffer RW1 to the column. Centrifuge for 15 s at 14 000 rpm at room temperature. Discard flow-through.
8. Add 10 µl RNase-free DNase (Qiagen) with 70 µl buffer RDD (Qiagen). Incubate at room temperature for 15 min.
9. Add 350 µl buffer RW1. Centrifuge for 15 s at 14 000 rpm at room temperature. Discard flow-through.
10. Transfer the column to a new collection tube and add 500 µl buffer RPE onto the column. Centrifuge for 15 s at 14 000 rpm at room temperature. Discard flow-through.
11. Add 500 µl buffer RPE to the column, and centrifuge for 2 min at 14 000 rpm at room temperature.
12. Place the column into a fresh 2 ml tube and centrifuge at 14 000 rpm, 1 min at room temperature.
13. Transfer the column to a 1.5 ml tube and elute using 30 µl of RNase-free water into the column, by centrifuging at 14 000 rpm for 1 min.
14. Put the same water (30 µl) through the column (step 13) and centrifuge at 14 000 rpm for 1 min.

# APPENDIX C. MOLECULAR WEIGHT MARKERS

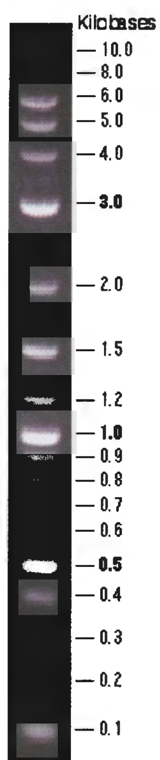
## C.1. Promega 1 kb DNA ladder



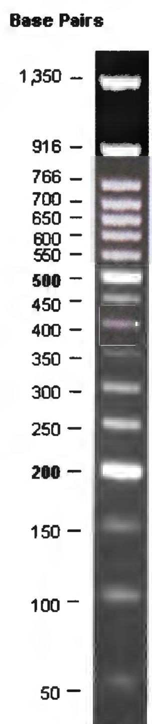

## C.2. New England Biolabs 100 bp DNA ladder



## C.3. New England Biolabs 2-log DNA ladder

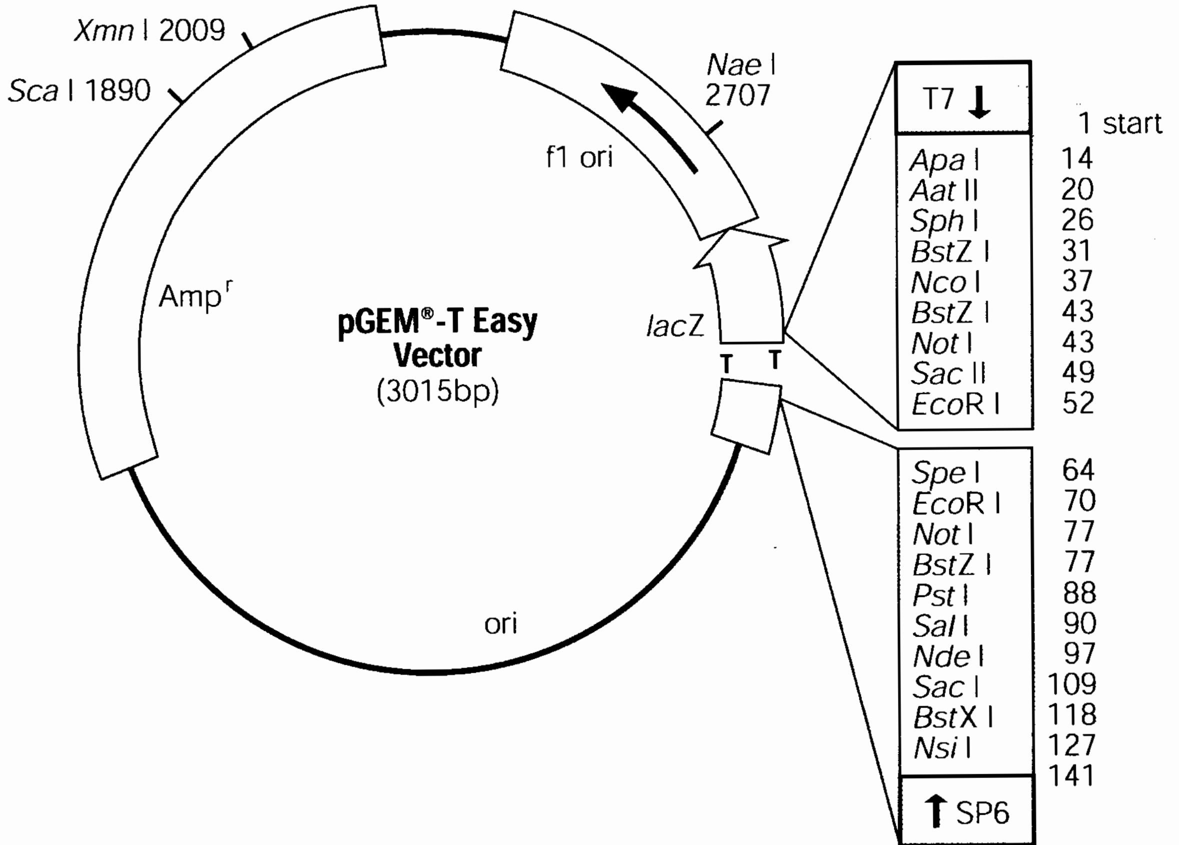



## C.4. New England Biolabs 50 bp DNA ladder



# APPENDIX D. PLASMIDS

## pGEM-T Easy Vector (Promega)




Amp$^r$ – ampicillin resistance gene coding for β-lactamase; *lacZ* – *lacZ* gene coding for β-galactosidase; T7 – T7 RNA polymerase promoter; SP6 – SP6 RNA polymerase promoter; ori – origin of replication; f1 ori – origin of replication of the filamentous phage f1.

# APPENDIX E. DATA COLLECTION SHEET

Patient No ..............................  Lab No SCH .................
Age .................  Sex ..............

Presenting symptoms

| | |
|---|---|
| Hemiparesis | ....... |
| Unsteady gait | ....... |
| Impaired visual acuity | ....... |
| Pupillary abnormalities | ....... |
| Personality changes | ....... |
| Mood disorder | ....... |
| Altered reflexes | ....... |
| Pain/photophobia | ....... |
| Diplopia | ....... |
| Other (specify) | .............................................. |
| | .............................................. |
| | .............................................. |

Duration of symptoms ..............................................

Results of other investigations

| | | |
|---|---|---|
| CSF | Polys | ....... |
| | Lymphs | ....... |
| | Protein | ....... |
| | Glucose | ....... |
| | Culture | ....... |
| | FTA | ....... |
| | VDRL | ....... |
| | Other | ........................... |
| | | ........................... |
| | | ........................... |
| Serum | FTA | ....... |
| | RPR | ....... |
| Other (specify) | ............................................ | |
| | ............................................ | |
| | ............................................ | |

Other diagnoses (suspected or confirmed)

..............................................................................
..............................................................................

Treatment with IV penicillin for neurosyphilis Y N

Follow up after treatment (if applicable) Y N

Outcome at follow up ............................................
............................................

# REFERENCES


ALDAVE, A.J., KING, J.A. & CUNNINGHAM, E.T. JR. 2001. Ocular syphilis. *Current opinion in ophthalmology*, 12(6):433-441.

APFALTER, P., ASSADIAN, O., BLASI, F., BOMAN, J., GAYDOS, C.A., KUNDI, M., MAKRISTATHIS, A., NEHR, M., ROTTER, M.L. & HIRSCHL, A.M. 2002. Reliability of nested PCR for detection of *Chlamydia pneumoniae* DNA in atheromas: results from a multicenter study applying standardized protocols. *Journal of clinical microbiology*, 40(12):4428-4434.

AUSUBEL, F.M., BRENT, R., KINGSTON, R.E., MOORE, D.D., SEIDMAN, J.G., SMITH, J.A. & STRUHL, K. 1987. Current protocols in molecular biology. Harvard Medical School: Green Publishing Associates and Wiley-Interscience.

BARKER, D.J.P. & ROSE, G. 1984. Epidemiology in medical practice. 3rd ed. New York: Churchill Livingstone. p. 122-130.

BIRNBOIM, H.C. & DOLY, J. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic acids research*, 7(6):1513-1523.

BLUM, L., BACHMEYER, C. & CAUMES, E. 2005. Seronegative secondary syphilis in an HIV-infected patient. *Clinical and experimental dermatology*, 30(2):158-159.

BONADIO, W.A. 1992. The cerebrospinal fluid: physiological aspects and alterations associated with bacterial meningitis. *The Pediatric infectious disease journal*, 11(6):1423-1432.

BURKE, J.M. & SCHABERG, D.R. 1985. Neurosyphilis in the antibiotic era. *Neurology*, 35(9):1368-1371.

BURSTAIN, J.M., GRIMPEL, E., LUKEHART, S.A., NORGARD, M.V. & RADOLF, J.D. 1991. Sensitive detection of *Treponema pallidum* by using the polymerase chain reaction. *Journal of clinical microbiology*, 29(1):62-69.

CASTRO, R.R., PRIETO, E.S., SANTO, I., AZEVEDO, J. & EXPOSTO, F.L. 2001. Evaluation of the passive particle agglutination test in the serodiagnosis and follow-up of syphilis. *American journal of clinical pathology*, 116(4):581-585.

CENTURION-LARA, A., CASTRO, C., SHAFFER, J.M., VAN VOORHIS, W.C., MARRA, C.M. & LUKEHART, S.A. 1997. Detection of *Treponema pallidum* by a sensitive reverse transcriptase PCR. *Journal of clinical microbiology*, 35(6):1348-1352.

CHAKRABORTY, R. & LUCK, S. 2007. Managing congenital syphilis again? The more things change... *Current opinion in infectious diseases*, 20(3):247-252.

CLINICAL AND LABORATORY STANDARDS INSTITUTE. 2006. Body fluid analysis for cellular composition: approved guideline. Wayne, PA. p. 67-70.

CLYNE, B. & JERRARD, D.A. 2000. Syphilis testing. *The journal of emergency medicine*, 18(3):361-367.

COGGON, D., ROSE, G. & BARKER, D.J.P. 1997. Epidemiology for the uninitiated. 4th ed. Plymouth: BMJ Publishing Group. p. 52-55.

COYNE, V.E., JAMES, M.D., REID, S.J. & RYBICKI, E.P. 2004. Molecular biology techniques manual. 7th ed. Cape Town, South Africa: Department of Molecular and Cell Biology, University of Cape Town. 125 p.

CUMMINGS, M.C., LUKEHART, S.A., MARRA, C., SMITH, B.L., SHAFFER, J., DEMEO, L., CASTRO, C. & McCORMACK, W.M. 1996. Comparison of methods for the detection of *Treponema pallidum* in lesions of early syphilis. *Sexually transmitted diseases*, 23(5):366-369.

DAGERT, M. & EHRLICH, S.D. 1979. Prolonged incubation in calcium chloride improves the competence of *Escherichia coli* cells. *Gene*, 6(1):23-28.

DAVIS, L.E. & SPERRY, S. 1979. The CSF-FTA test and the significance of blood contamination. *Annals of neurology*, 6(1):68-69.

DEACON, W.E., LUCAS, J.B. & PRICE, E.V. 1966. Fluorescent treponemal antibody-absorption (FTA-ABS) test for syphilis. *The journal of the American Medical Association*, 198(6):624-628.

DEPARTMENT of Health ***see*** SOUTH AFRICA. Department of Health.

DEPARTMENT of Health and Human Services ***see*** UNITED STATES OF AMERICA. Department of Health and Human Services.

ECKERT, K.A. & KUNKEL, T.A. 1990. High fidelity DNA synthesis by *Thermus aquaticus* DNA polymerase. *Nucleic acids research*, 18(13):3739-3744.

ERLICH, H.A., GELFAND, D. & SNINSKY, J.J. 1991. Recent advances in the polymerase chain reaction. *Science*, 252(5013):1643-1651.

ESCOBAR, M.R., DALTON, H.P. & ALLISON, M.J. 1970. Fluorescent antibody tests for syphilis using cerebrospinal fluid: clinical correlation in 150 cases. *American journal of clinical pathology*, 53(6):886-90.

FINNZYMES. 2005a. DyNAzyme EXT DNA Polymerase. Reagents kit insert (version 2.0).

FINNZYMES. 2005b. RobusT II RT-PCR Kit instruction manual.

FUJIREBIO INC. 2006. Serodia-TP-PA: reagents for the detection of antibodies to *Treponema pallidum*. Reagents kit insert.

GIESSE DIAGNOSTICS. 2005. TPHA microplate hemagglutination: for detection of anti-*Treponema pallidum* antibodies. Reagents kit insert.

GOLDMEIER, D. & GUALLAR, C. 2003. Syphilis: an update. *Clinical medicine*, 3(3):209-211.

GRIMPREL, E., SANCHEZ, P.J., WENDEL, G.D. JR, BURSTAIN, J.M., McCRACKEN, G.H. JR, RADOLF, J.D. & NORGARD, M.V. 1991. Use of polymerase chain reaction and rabbit infectivity testing to detect *Treponema pallidum* in amniotic fluid fetal and neonatal sera and cerebrospinal fluid. *Journal of clinical microbiology*, 29(8):1711-1718.

GRUNENWALD, H. 2003. Optimization of polymerase chain reactions. (*In* BARTLETT, J.M.S. & STIRLING, D., *eds.* 2nd ed. PCR protocols. Totowa, NJ: Humana Press Inc. p. 89-99.)

HART, G. 1986. Syphilis tests in diagnostic and therapeutic decision making. *Annals of internal medicine*, 104(3):1368-1371.

HAY, P.E., CLARKE, J.R., STRUGNELL, A., TAYLOR-ROBINSON, D. & GOLDMEIER, D. 1990. Use of polymerase chain reaction to detect DNA sequences specific to pathogenic treponemes in cerebrospinal fluid. *FEMS microbiology letters*, 68(3):233-238.

HEYMANN, W.R. 2006. The history of syphilis. *Journal of the American Academy of Dermatology*, 54(2):322-323.

HICKS, C.B., BENSON, P.M., LUPTON, G.P. & TRAMONT, E.C. 1987. Seronegative secondary syphilis in a patient infected with the human immunodeficiency virus (HIV) with Kaposi sarcoma. *Annals of internal medicine*, 107(4):492-495.

HIGUCHI, R. 1989. Simple and rapid preparation of samples for PCR. (*In* ERLICH, H.A., *ed.* PCR technology: principles and applications for DNA amplification. New York: Stockton Press Ltd. p. 31-38.)

HOOK, E.W. III & MARRA, C.M. 1992. Acquired syphilis in adults. *The New England journal of medicine*, 326(16):1060-1090.

HOOK, E.W. III, RODDY, R.E., LUKEHART, S.A., HOM, J., HOLMES, K.K., TAM, M.R. 1985. Detection of *Treponema pallidum* in lesion exudate with a pathogen-specific monoclonal antibody. *Journal of clinical microbiology,* 22(2):241-244.

HYNDMAN, D.L. & MITSUHASHI, M. 2003. PCR primer design. (*In* BARTLETT, J.M.S. & STIRLING, D., *eds.* 2nd ed. PCR protocols. Totowa, NJ: Humana Press Inc. p. 81-88.)

ISH-HOROWICZ, D. & BURKE, J.F. 1981. Rapid and efficient cosmid cloning. *Nucleic acids research*, 9(13):2989-2998.

JAFFE, H.W., LARSEN, S.A., PETERS, M., JOVE, D.F., LOPEZ, B. & SCHROETER, A.L. 1978. Tests for treponemal antibody in CSF. *Archives of internal medicine*, 138(2):252-255.

JOHNS, D.R., TIERNY, M. & FELSENSTEIN, D. 1987. Alteration in the natural history of neurosyphilis by concurrent infection with the human immunodeficiency virus. *The New England journal of medicine*, 316(25):1568-1572.

KATZ, D.A. & BERGER, J.R. 1989. Neurosyphilis in acquired immunodeficiency syndrome. *Archives of neurology*, 46(8):895-898.

KAWASAKI, E.S. & WANG, A.M. 1989. Detection of gene expression. (*In* ERLICH, H.A., *ed.* PCR technology: principles and applications for DNA amplification. New York: Stockton Press Ltd. p. 89-97.)

KINGSTON, A.A., VUJEVICH, J., SHAPIRO, M., HIVNOR, C.M., JUKIC, D.M., JUNKINS-HOPKINS, J.M., JIH, D.M., KOSTMAN, J.R. & JAMES, W.D. 2005. Seronegative secondary syphilis in 2 patients coinfected with human immunodeficiency virus. *Archives of dermatology*, 141(4):431-433.

KLETER, B., VAN DOORN, L.J., TER SCHEGGET, J., SCHRAUWEN, L., VAN KRIMPEN, K., BURGER, M., TER HARMSEL, B. & QUINT, W. 1998. Novel short-fragment PCR assay for highly sensitive broad-spectrum detection of anogenital human papillomaviruses. *The American journal of pathology*, 153(6):1731-1739.

KUO, K., LEUNG, M.F.K.L. & LEUNG, W. 1997. Intrinsic secondary structure of human TNFR-1 mRNA influences the determination of gene expression by RT-PCR. *Molecular and cellular biochemistry*, 177(1-2):1-6.

LAFOND, R.E. & LUKEHART, S.A. 2006. Biological basis for syphilis. *Clinical microbiology reviews*, 19(1):29-49.

LARSEN, S.A., STEINER, B.M. & RUDOLPH, A.H. 1995. Laboratory diagnosis and interpretation of tests for syphilis. *Clinical microbiology reviews*, 8(1):1-21.

LECLERC, G., GIROUX, M., BIRRY, A. & KASATIYA, S. 1978. Study of fluorescent treponemal antibody test on cerebrospinal fluid using monospecific anti-immunoglobulin conjugates IgG, IgM and IgA. *The British journal of venereal diseases*, 54(5):303-308.

LEE, J.W., WILCK, M. & VENNA, N. 2005. Dementia due to neurosyphilis with persistently negative CSF VDRL. *Neurology*, 65(11):1838.

LEVINE, S.M., PEREZ-PEREZ, G., OLIVARES, A., YEE, H., HANNA, B.A. & BLASER, M.J. 2002. PCR-based detection of *Bacillus anthracis* in formalin-fixed tissue from a patient receiving ciprofloxacin. *Journal of clinical microbiology*, 40(11):4360-4362.

LIU, H., RODES, B., CHEN, C.Y. & STEINER, B. 2001. New tests for syphilis: rational design of a PCR method for detection of *Treponema pallidum* in clinical specimens using unique regions of the DNA polymerase I gene. *Journal of clinical microbiology*, 39(5):1941-1946.

LOUIE, M., LOUIE, L. & SIMOR, A.E. 2000. The role of DNA amplification technology in the diagnosis of infectious diseases. *Canadian Medical Association journal*, 163(3):301-309.

LUKEHART, A.S., HOOK, E.W., BAKER-ZANDER, S.A., COLLIER, A.C., CRITCHLOW, C.W. & HANDSFIELD, H.H. 1988. Invasion of the central nervous system by *Treponema pallidum*: implications for diagnosis and treatment. *Annals of internal medicine*, 109(11): 855-862.

MANIATIS, T., FRITSCH, E.F. & SAMBROOK, J. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Press.

MARGO, C.E. & HAMED, L.M. 1992. Ocular syphilis. *Survey of ophthalmology*, 37(3):203-220.

MATSUMOTO, M., ISHIKAWA, F., MATSUBAYASHI, T., NAGAO, T., INUZUMI, K., ARAKI, H., KAWAI, K. & OSATO, K. 1993. Latex agglutination test for detecting antibodies to *Treponema pallidum*. *Clinical chemistry*, 39(8):1700-1705.

MILLER, J.H. 1992. A short course in bacterial genetics: a laboratory manual and handbook for *Escherichia coli* and related bacteria. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press. p. 57 – 62.

MUSHER, D.M., HAMILL, R.J. & BAUGHN, R.E. 1990. Effect of human immunodeficiency virus (HIV) infection on the course of syphilis and on the response to treatment. *Annals of internal medicine*, 113(11):872-881.

NOORDHOEK, G.T., WOLTERS, E.C., DE JONGE, M.E.J. & VAN EMBDEN, J.D.A. 1991. Detection by polymerase chain reaction of *Treponema pallidum* DNA in cerebrospinal fluid from neurosyphilis patients before and after antibiotic treatment. *Journal of clinical microbiology*, 29(9):1976-1984.

ORLE, K.A., GATES, C.A., MARTIN, D.H., BODY, B.A. & WEISS, J.B. 1996. Simultaneous PCR detection of *Haemophilus ducreyi*, *Treponema pallidum* and herpes simplex virus types 1 and 2 from genital ulcers. *Journal of clinical microbiology*, 34(1):49-54.

PEDERSEN, N.S., ØRUM, O. & MOURITSEN, S. 1987. Enzyme-linked immunosorbent assay for detection of antibodies to venereal disease research laboratory (VDRL) antigen in syphilis. *Journal of clinical microbiology*, 25(9):1711-1716.

PLASMATEC. 2003. TPHA test kit. Reagents kit insert.

POPE, V., FEARS, M.B., MORRILL, W.E., CASTRO, A. & KIKKERT, S.E. 2000. Comparison of the Serodia *Treponema pallidum* Particle Agglutination, Captia Syphilis-G, and SpiroTek Reagin II tests with standard test techniques for diagnosis of syphilis. *Journal of clinical microbiology*, 38(7):2543-2545.

PROMEGA. 2003. pGEM-T and pGEM-T Easy Vector Systems: technical manual no. 042.

QIAGEN. 2001. RNeasy Mini Handbook (third edition).

QIAGEN. 2002. QIAquick Spin Handbook.

QIAGEN. 2006. RNAlater Handbook.

RATNAMOHAN, V.M., CUNNINGHAM, A.L. & RAWLINSON, W.D. 1998. Removal of inhibitors of CSF-PCR to improve diagnosis of herpesviral encephalitis. *Journal of virological methods*, 72(1):59-65.

ROMANOWSKI, B., FORSEY, E., PRASAD, E., LUKEHART, S., TAM, M. & HOOK, E.W. 1987. Detection of *Treponema pallidum* by a fluorescent monoclonal antibody test. *Sexually transmitted diseases*, 14(3):156-159.

SAMBROOK, J., FRITSCH, E.F. & MANIATIS, T. 1989. Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Press.

SCHNAITMAN, C.A. 1971. Effect of ethylenediaminetetraacetic acid, Triton X-100, and lysozyme on the morphology and chemical composition of isolated cell walls of *Escherichia coli*. *Journal of bacteriology*, 108(1):553-563.

SINGH, A.E. & ROMANOWSKI, B. 1999. Syphilis: review with emphasis on clinical, epidemiologic, and some biologic features. *Clinical microbiology reviews*, 12(2):187-209.

STRYER, L. 1995. Biochemistry. 4th ed. New York: W.H. Freeman and Company. p. 17-74, 119-144, 787-818.

SOUTH AFRICA. Department of Health. 2005. National HIV and syphilis antenatal sero-prevalence survey in South Africa 2004. Pretoria: Government Printer. 18 p.

TELLIER, R., BUKH, J., EMERSON, S.U. & PURCELL, R.H. 2003. Long PCR methodology. (*In* BARTLETT, J.M.S. & STIRLING, D., *eds.* 2nd ed. PCR protocols. Totowa, NJ: Humana Press Inc. p. 173-177.)

TOMBERLIN, M.G., HOLTOM, P.D., OWENS, J.L. & LARSEN, R.A. 1994. Evaluation of neurosyphilis in human immunodeficiency virus-infected individuals. *Clinical infectious diseases*, 18(3):299-294.

TRAMONT, E.C. 2000. *Treponema pallidum* (syphilis). (*In* MANDELL, G.L., BENNET, J.E., DOLIN, R., *eds*. 5th ed. Mandell, Douglas and Bennet's principles and practice of infectious disease. PA: Churchill Livingstone. p. 2474 – 2490.)

TURNER, T.B., HARDY, P.H. & NEWMAN, B. 1969. Infectivity tests in syphilis. *The British journal of venereal diseases*, 45(3):183-196.

ULLMANN, A., JACOB, F. & MONOD, J. 1967. Characterization by *in vitro* complementation of a peptide corresponding to an operator-proximal segment of the beta-galactosidase structural gene of *Escherichia coli. Journal of molecular biology*, 24(2):339-343.

UNITED STATES OF AMERICA. Department of Health and Human Services, Centers for Disease Control and Prevention. 2006. Sexually transmitted disease surveillance 2005 supplement, syphilis surveillance report. USA: US Government Printing Office. 74 p.

VERSALOVIC, J. & LUPSKI, J.R. 2002. Molecular detection and genotyping of pathogens: more accurate and rapid answers. *Trends in microbiology*, 10(10 Suppl): S15-S21.

WIAME, I., REMY, S., SWENNEN, R. & SÁGI, L. 2000. Irreversible heat inactivation of DNase I without RNA degradation. *Biotechniques*, 29(2):252-256.

WICHER, K., HOROWITZ, H.W. & WICHER, V. 1999. Laboratory methods of diagnosis of syphilis for the beginning of the third millennium. *Microbes and infection*, 1(12):1035-1049.

YOUNG, H. 1998. Syphilis: serology. *Dermatologic clinics*, 16(4):691-698.