CALYMMATOBACTERIUM GRANULOMATIS :

CULTURE, ELECTRON MICROSCOPIC STUDIES

AND

MOLECULAR ANALYSIS

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ABSTRACT

Granuloma inguinale has emerged as a significant cause of genital ulcer disease (GUD) in the KwaZulu/Natal region of South Africa. The aetiological agent *Calymmatobacterium granulomatis,* has been poorly studied, because of its fastidious nature and the inability to cultivate it.

This study describes the first ever successful culture of *C. granulomatis* in peripheral blood monocytes (PBMNC), its ultrastructural characteristics and molecular analysis of the 16S ribosomal RNA gene sequence of the organism.

Initially, tissue biopsy specimens from female patients with clinical and laboratory confirmed diagnosis of granuloma inguinale were treated with amikacin (10mg/L) and inoculated in a monocyte co-culture system using peripheral blood mononuclear cells (PBMNC) and autologous serum from a single donor. This method was modified by the addition of vancomycin (5 mg/L) and metronidazole (10 mg/L) to amikacin for decontamination and by the use of pooled PBMNC from normal blood donors supplemented with heat inactivated foetal calf serum for co-culture. The modified method was used to culture biopsy specimens as well as ulcer scrapings from female and male patients with GUD. All monocyte co-cultures were examined by a rapid Giemsa (RapiDiff) stain and by an indirect immunofluorescence test. Representative monocyte co-cultures, frozen and paraffin wax embedded biopsy specimens were examined by transmission electron microscopy for to ascertain the ultrastructural characteristics and the isolation, amplification and sequencing of the 16S rRNA gene was undertaken.

C. granulomatis was isolated in pure culture from six biopsy specimens and fourteen genital ulcer scrapings. On light microscopy the cultured organisms were visualised intra and extracellularly. After 48 hours incubation the organism appeared elongated with tapered or bulging ends. The bacilli displayed intensely staining single and bipolar condensation and a faint outline. After subsequent passages, the bacilli were extremely pleomorphic with the single and bipolar condensation still evident. The presence of a halo around the organism in some cultures indicated a capsule. When using older monocytes for the

primary isolation of *C. granulomatis*, the organism was predominantly intracellular. In the subcultures the bacteria were intracellular, appearing singly within a single vacuole or as a cluster of bacilli within a vacuole. The initial growth of the organism was slow in the first 24 hours, increased over the next 48 hours and declined after 96 hours. The number of organisms in the monocyte co-culture increased after each passage and these were maintained for eight passages. All positive monocyte co-cultures showed bright fluorescence when tested with immune sera.

The ultrastructural morphological features of the organism in the monocyte co-cultures were similar to those seen in tissue biopsy specimens. In the former, the bacteria were extracellular and not within membrane bound vacuoles, whilst in the tissue they were located predominantly within vacuoles of varying sizes in the cytoplasm of the macrophages and occasionally, extracellularly in the intercellular spaces of the stroma. The cellular response consisted of macrophages, polymorphonuclear neutrophils and plasma cells. The cytoplasm of the bacteria contained ribosomes scattered throughout the bacterial body and electron dense granules positioned towards the periphery. The trilaminar cell wall structure was that of a Gram negative organism consisting of an outer membrane, a middle electron opaque layer and an inner plasma membrane. The bacterial body in the monocyte co-culture was surrounded by a uniformly extensive homogeneous layer of high electron contrast, whilst this layer in the bacteria from the tissues specimens varied considerably being homogeneously electron dense to delicate web like structures with varying density and thickness. Surface structures such as fimbriae, flagellae and bacteriophage were not identified.

The 16S rRNA gene sequence of *C. granulomatis* from the monocyte co-culture, frozen and formalin fixed paraffin wax embedded biopsy specimens was determined. Analysis of the sequences in the three specimens were found to be identical with sequence homology of >99%. The differences occurred in 13 base pair positions. A comparative analysis of rRNA sequences of similar bacteria showed that the organism formed a distinct group and was closely related to the *Klebsiella* group of the taxon *Enterobacteriacaeae.* The sequence signatures classified the organism to the gamma subclass of *Proteobacteria.* On the basis of the results of the phylogenetic analysis, it is proposed that the classification of the genus

Calymmatobacterium be retained and the organism not be placed in the *Klebsiella* group.

Hybridisation experiments performed using specific universal bacterial (RDR245) and Gram negative (DLO4) oligonucleotide probes suggested that the restricted fragments from the recombinant clones from all three sources were Eubacterial in origin. Conclusive proof that the organisms were Gram negative and not Gram positive was the fact that no hybridisation signal was obtained with the Gram positive probe (RWO3).

This study reports the development of the first ever tissue culture system (monocyte co-culture) for the cultivation of *C. granulomatis*. Absence of growth of any contaminating organism/s on cell free media, increase in numbers in the co-culture, demonstration of fluorescence with immune sera, the ultrastructural characteristics and 16S rRNA gene sequence analysis confirm the growth of this previously uncultivated organism. Furthermore, the sequence analysis confirms the placement of *Calymmatobacterium* in the gamma subclass within the *Proteobacteria* group.

To my beloved

Children

Irshaad, Zahira and Khadija

for their patience and tolerance which they had to endure at their tender age.

PREFACE

This study represents original work by the author and has not been submitted in any form to another University. Where use was made of the work of others it has been duly acknowledged in the text.

The research described in this thesis was performed in the Department of Medical Microbiology, Faculty of Medicine, University of Natal, under the supervision of Dr Anwar A Hoosen and Dr Photini Kiepiela.

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PUBLICATIONS

- Culture of *Calymmatobacterium granulomatis*
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  Clinical Infectious Diseases 1996; 22 : 391.

- Ultrastructural study of *Calymmatobacterium granulomatis* : the causative agent of granuloma inguinale
  ABM Kharsany, AA Hoosen, P Kiepiela, T Naicker, AW Sturm
  Proceedings of the Microscopy Society of Southern Africa 1996; 26 : 89.

- Growth and cultural characteristics of *Calymmatobacterium granulomatis* - the aetiological agent of granuloma inguinale (Donovanosis)
  ABM Kharsany, AA Hoosen, P Kiepiela, T Naicker, AW Sturm
  Journal of Medical Microbiology 1997; 46 : 579 - 585.

- Comparative ultrastructural morphology of *Calymmatobacterium granulomatis* from from cultured and tissue biopsy specimens
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- Sequence analysis of *Calymmatobacterium granulomatis* - the aetiological agent of granuloma inguinale (Donovanosis)
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  Submitted for publication

PRESENTATIONS AT SCIENTIFIC MEETINGS

- Culture of *Calymmatobacterium granulomatis* - a preliminary report
  ABM Kharsany, AA Hoosen, P Kiepiela, D York, T Naicker, AW Sturm
  The Federation of South African Societies of Pathology, 34th Annual Congress, Cape Town 3 - 6 July 1994.

- Culture of *Calymmatobacterium granulomatis* - a preliminary report
  ABM Kharsany, AA Hoosen, P Kiepiela, D York, T Naicker, AW Sturm
  Faculty Research Day, University of Natal, 7 September 1994.
  PRIZE FOR BEST PAPER PRESENTATION.

- *Calymmatobacterium granulomatis : in - vitro* culture and molecular characterisation
  ABM Kharsany, AA Hoosen, P Kiepiela, D York, T Naicker, AW Sturm
  5th Joint Congress of the Sexually Transmitted Diseases and Infectious Diseases Societies of southern Africa, Durban 20 - 24 May 1995.

- *Calymmatobacterium granulomatis: in - vitro* culture and molecular characterisation
  ABM Kharsany, AA Hoosen, P Kiepiela, D York, T Naicker, AW Sturm
  Eleventh meeting of the International Society for STD Research, New Orleans, Louisiana, 27 - 30 August, 1995.

- Ultrastructural study of *Calymmatobacterium granulomatis* : the causative agent of granuloma inguinale
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- *Calymmatobacterium granulomatis* is a member of the gamma subgroup of Gram negative bacteria
  ABM Kharsany, AA Hoosen, P. Kiepiela, R Kirby, A.W. Sturm.
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AIDS	acquired immunodeficiency syndrome
A, C, G, T	adenosine, cytosine, guanine, thymine
bp	base pairs
BCIP	5-bromo-4-chloro-3-indolyl phosphate
C	celsius
cm	centimeters
CO_2	carbon dioxide
dH_2O	distilled water
DIG	digoxygenin
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EDTA	ethylene diamine tetra acetic acid
et al	*et alii* (and others)
G + C	guanine + cytosine
g/l	grams per litre
HBSS	Hanks' balanced salt solution
HCl	hydrochloric acid
HIV	human immunodeficiency virus
IPTG	isopropylthio-β-D-galactoside
kb	kilobase
kDa	kilodalton
LB	Lauria Bertoni
M	molar
mag	magnification
mg	milligram/s
mg/L	milligram/s per litre
$MgCl_2$	magnesium chloride
ml	millilitre/s
mm	millimetre/s
mM	millimole
MW	molecular weight
NaCl	sodium chloride
NaOH	sodium hydroxide
NBT	nitroblue tetrazolium
nm	nanometer
OD	optical density
PBMNC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
pmoles	picomoles
rDNA	ribosomal deoxyribonucleic acid
rRNA	ribosomal ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulphate

SSC	sodium chloride, sodium citrate
STD	Sexually transmitted disease/s
TBE	tris-borate EDTA
TE	tris-HCL-EDTA
U	unit
µg	microgram/s
µl	microlitre/s
µm	micrometre/s
µg/ml	microgram/s per millilitre
UV	ultraviolet
v/v	volume per volume
V	volt
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-galactoside

CHAPTER 1.0
GENERAL INTRODUCTION

Sexually transmitted diseases (STDs) are a group of communicable diseases which have the common feature of being spread predominantly by sexual contact. Worldwide, STDs constitute a major public health problem on account of their frequency, occurrence, effect on maternal and child health, social consequences, economic costs in terms of health expenditure and loss of productivity. In most developing countries, STDs have reached unprecedented levels due to the lack of effort to contain the spread and therefore any hopes to contain their spread has not been realised. This is directly related to the lack of economic resources, medical personnel and diagnostic facilities. Furthermore, with very little funding available in developing countries, the management and control of STD's has not been prioritised. This has resulted in considerable variation in the patterns and presentation of most STDs (Adler, 1996; Mabey, 1996).

Reliable data on the prevalence and incidence of STDs in developed countries are available. Syphilis and gonorrhoeae were the commonest STDs in the United States of America and reached a peak in the late 1940's, however, with the wider use of antibiotics and careful contact tracing, lowest rates were recorded in the 1950's. Since then there has been a gradual increase in the incidence of STDs (Evans, 1976a). For the developing countries, especially in Africa very little reliable information is available regarding the prevalence and patterns of sexually transmitted diseases. The general impression appears to be that gonorrhoea, syphilis and chancroid are hyperendemic in urban areas of many tropical African countries, spreading to rural areas (Osoba, 1981; Mabey, 1966).

In South Africa, statistics on the incidence and prevalence of STDs have been variable, yet reports confirm very high rates for most STDs (Hoosen *et al,* 1979; Duncan *et al,* 1981; Coovadia *et al,* 1985; Pham-Kanter *et al*, 1996). Presently adequate facilities for the appropriate diagnosis and treatment of STDs are grossly lacking. The City Health Municipal STD Clinic in the metropolitan region of Durban is the only designated clinic for the diagnosis and treatment of sexually transmitted diseases serving the indigent Black

community. Evidence of an increase in the incidence of sexually transmitted diseases in this region is available from the Medical Officer of Health's annual reports. For the year 1995 a total of 65 000 cases were seen, of which approximately 45 000 were new cases. The total number of patients seeking treatment for an ulcerative condition was almost 21 000, of which 17 000 were males and 4 000 females (Figure 1). These figures are considerably higher than those seen five to ten years ago (Figure 1; O'Farrell, 1992).

All strata of societies are affected by STDs, however the patterns differ with some infections appearing to be commoner in certain ethnic groups. In developed countries genital ulcer disease is considered to be a relatively minor problem with herpes genitalis being the commonest cause, whilst in developing countries, the genital ulcers are a most common manifestation of STDs, with chancroid and syphilis being the commonest causes. The sexually transmitted diseases which commonly present as genital ulcerations are those listed in table I (Duncan *et al*, 1981).

Granuloma inguinale was considered to be uncommon in South Africa. It was recognised in 1916 (Ricono, 1916), and sporadically reported until the 1980's. Several cases were reported from the Transvaal (Gauteng) (Duncan *et al*,1981), Eastern Transvaal (Mpumalanga) (Wistrand and Wegerhoff, 1985) and Natal (Kwa/Zulu Natal) (Coovadia *et al,* 1985). Since these reports there has been an increase in the awareness of the disease and more cases have been diagnosed and reported from the Kwa/Zulu Natal region (Hoosen *et al,* 1990; O'Farrell, 1990, 1991a, 1991b, 1992, 1993; Bassa *et al*, 1992; Hoosen *et al,* 1996).

Accurate statistics on the prevalence of granuloma inguinale, both in the community and hospital are not readily available. In 1992 O'Farrell reported on the prevalence of 313 cases of granuloma inguinale in a STD clinic population for the year 1988. Since then there has been a consistent gradual increase in the number of reported cases. The medical officers annual reports show a significant increase in the number of new cases of granuloma inguinale at the Sexually Transmitted Diseases clinic in Durban, for the years 1988 to 1995 (Figure 2). Thus granuloma inguinale is being reported with increasing frequency especially in the Kwa/Zulu Natal region of South Africa.

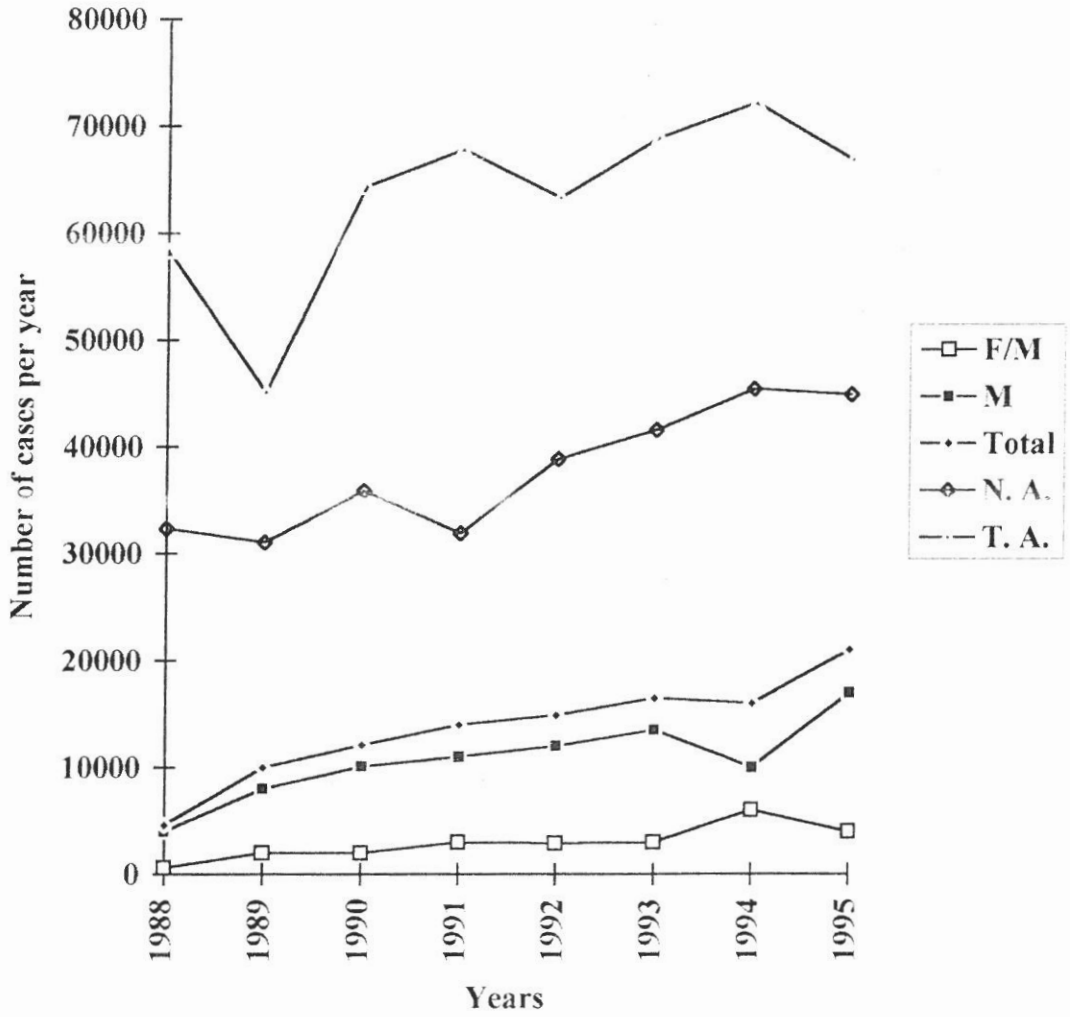


Figure 1: Sexually transmitted diseases clinic attenders. Numbers of cases per year of total (T.A), new attenders (N.A) and total (total), new cases of males (M) and females (F/M) patients presenting with ulcerative conditions at the Sexually Transmitted Diseases clinic (Durban) as recorded in the annual reports of the Medical Officer of Health for the years 1988 to 1995.

TABLE I: SEXUALLY TRANSMITTED DISEASES WHICH COMMONLY PRESENT AS GENITAL ULCERATIONS (DUNCAN *et al*, 1981)

Aetiological agent	**Clinical condition**
Treponema pallidum	Syphilis
Haemophilus ducreyi	Chancroid
Herpes simplex virus types 1 & 2 (usually type 2)	Herpes genitalis
Chlamydia trachomatis (Serotype L1, L2 and L3)	Lymphogranuloma venereum (LGV)
Calymmatobacterium granulomatis	Granuloma inguinale (Donovanosis)

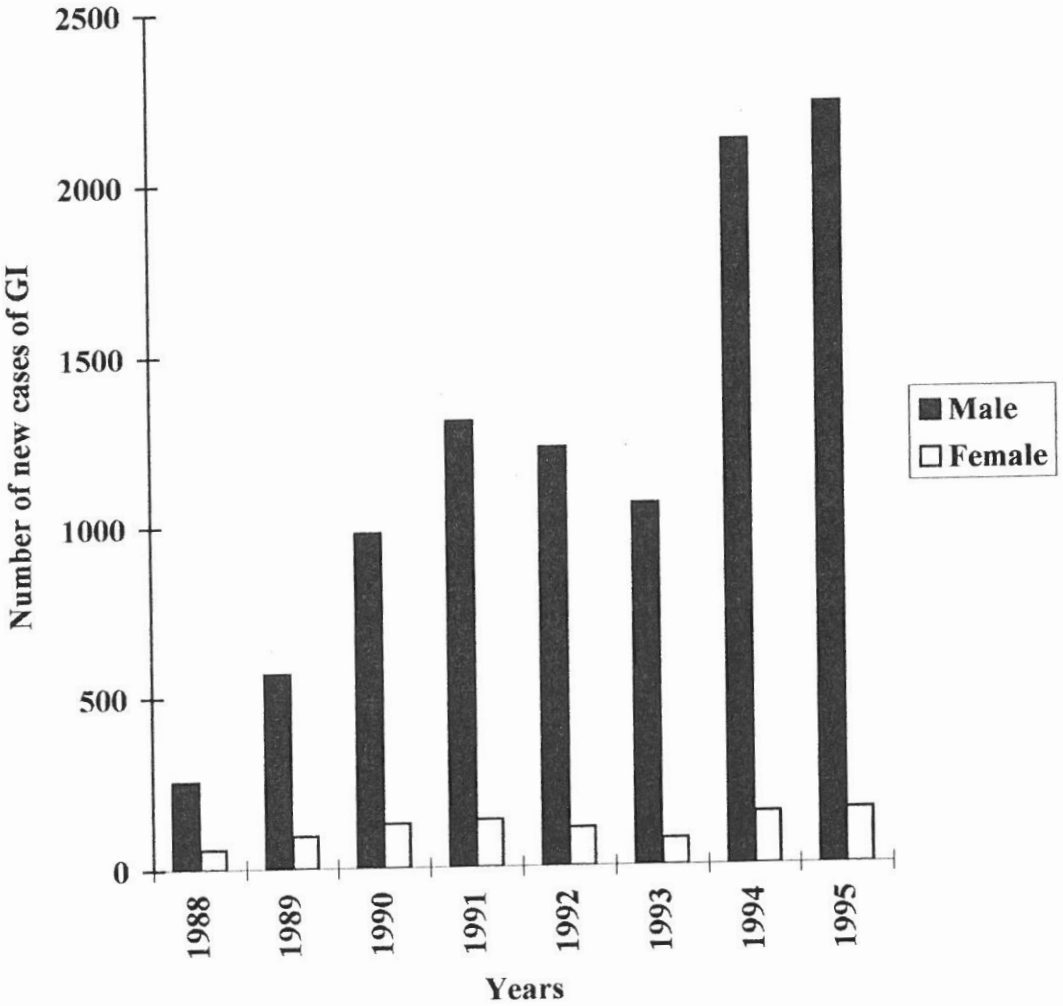


Figure 2: Number of granuloma inguinale cases recorded in the Durban metropolitan region for the years 1988 to 1995. Extracted from the annual reports of the Medical Officer of Health, Durban.

Since the emergence of the human immunodeficiency virus (HIV) the clinical course, presentations and response to therapy for certain ulcerative conditions have been affected (Wasserheit, 1992). STD clinic attenders form an important group of patients who act as a source for the increased risk of acquisition or transmission of HIV infection. In the presence of both ulcerative and non-ulcerative STDs there is an increase in the risk of HIV transmission, whilst genital ulcer disease is a major co-factor in the spread of the HIV, with a risk of greater than eight fold (Greenblatt *et al,* 1987; Wasserheit, 1992).

The lesions are characteristically extensive and bleed easily. They are often painless and therefore do not preclude sexual intercourse. In a study reported by O'Farrell *et al,* (1992) 44% of men and 80% of women with granuloma inguinale continued with sexual intercourse despite the presence of genital lesions. The potential role of genital ulcer disease, especially granuloma inguinale in the acquisition and transmission of the human immunodefieciency virus is of concern as it is well known that STDs and HIV infections interact in a bidirectional and mutually enhancing manner (Wasserheit, 1992, Clottey and Dallabetta, 1993).

The organism implicated in the aetiology of granuloma inguinale, *Calymmatobacterium granulomatis,* has consistently been observed as Donovan bodies within the macrophages in the lesions. Although discovered more than 90 years ago, its characterisation has been hampered because of the inability to cultivate the organism *in vitro.* The organism has rarely been maintained in the laboratory. Cultivation of the bacterium was restricted in the yolk sac of fertile chick eggs more than 30 years ago (Anderson, 1943; Anderson *et al*, 1945a) and therefore the lack any reference cultures. To date there is no information on the genetics of *C. granulomatis* and therefore the use of molecular biological techniques to study or characterise the organism has not been explored.

Recently, the use of DNA techniques has circumvented the problems encountered in identifying a large number of "culture resistant" bacteria (Wilson, 1994) on the basis of the amplification and sequence analysis of the 16S ribosomal RNA gene of microorganisms directly from infected tissue material. Many microbial pathogens have been identified using these techniques and to establish causation either the ribosomal RNA sequence obtained

should be that of a known pathogen (Wilson, 1994) or the true previously identified infectious agent should be found at the site of infection in multiple patients with a given infectious process (Relman *et al*, 1990).

AIMS OF THE STUDY

the aims of this study were to:

- establish an *in vitro* culture method for the growth and propagation of the aetiological agent of granuloma inguinale - *Calymmatobacterium granulomatis*

- determine the morphological characteristics of the organism in culture and in tissue biopsy specimens

- to sequence the 16s ribosomal RNA gene of *Calymmatobacterium granulomatis* from culture and directly from tissue biopsy specimens and to compare these sequences.
- to characterise *Calymmatobacterium granulomatis* on the basis of culture morphology, ultrastructural characteristics and sequence analysis.

CHAPTER 2.0
LITERATURE REVIEW

2.1 INTRODUCTION

Granuloma inguinale (Donovanosis) is a chronic progressively destructive disease of venereal origin. It is characterised by "granulomatous" ulcerations of the genitalia and surrounding sites with little or no tendency to spontaneous healing (Rajam and Rangiah, 1954). To date this description remains classic for the infection.

The clinical lesions of granuloma inguinale were first described in Madras, India by McLeod in 1882, whilst Conyers and Daniels (1896 - cited by Rajam and Rangiah, 1954) reported on cases from British Guyana and gave a good clinical description of the disease. In 1897, Galloway presented a detailed description of the lesion from a single case seen in London, in a native of the West Indies. No aetiological agents were ascribed to these conditions.

The clinical condition has been described by a number of names such as, serpiginous ulceration of the genitalia (McLeod, 1882), form of groin ulceration (Conyers and Daniels, 1896 - cited by Rajam and Rangiah, 1954), ulcerating granuloma of the pudenda (Galloway, 1897), chronic venereal sores (Maitland, 1898), infectious granuloma, granuloma inguinale tropicum (Crocker, 1903 - cited by Marmell and Santora, 1950), granuloma genito-inguinale (Nair and Pandalai, 1934), granuloma venereum genito-inguinale (Chettiar and Mathuswami, 1935 - cited by Marmell and Santora, 1950), granuloma inguinale, granuloma venereum, ulcerating sclerosing granuloma (Brooke, 1928) and granuloma Donovani (Lal and Nicholas, 1970). The disease is now commonly known as granuloma inguinale or Donovanosis (Marmell and Santora, 1950).

It was not until 1905 that Donovan in Madras, India (Donovan, 1904; 1905) recognised and described the presence of intracellular bodies from an oral lesion of a patient under his care. He considered these bodies to be protozoan in nature. Thereafter, an additional six cases

were reported together with the description of the causative organism observed in large mononuclear cells. The appearance was described as resembling "the gregarini form stage of a herpetomonas or crithidium" (Carter 1910). In 1913 Aragao and Vianna proposed the name *Calymmatobacterium granulomatis* for the organisms which were classified as bacteria and later given the name *Donovania granulomatis* by Anderson *et al* (1945a). The classical description of the disease together with the claims of having cultured an intracellular capsulated bacillus and the recognition of the "bodies" in histological sections of the diseased tissue were reported in the USA by Goldzieher and Peck (1926).

The specific histological appearance of granuloma inguinale consisting of large mononuclear cells filled with intracytoplasmic vacuoles containing deeply staining bodies, being the pathognomonic cells of the disease were described by Pund and Greenblatt in 1937. The clinical condition was reproduced in a human volunteer by the subcutaneous injection of Donovan bodies from pus containing the organisms aspirated from a patient with a pseudobubo (Greenblatt *et al* 1939).

Although *Calymmatobacterium granulomatis* has been associated with granuloma inguinale it's role in the causation of granuloma inguinale was uncertain (Castellani and Mendelson, 1929). Several workers claimed to have cultured the organism in artificial cell free media (McIntosh, 1926; DeMonbreun and Goodpasture, 1931b), but this has not been reproducible. Reports on the cultivation of the Donovan bodies in the yolk sac of developing chick embryo were made by Anderson (Anderson, 1943; Anderson *et al,* 1945a). Following this technique for primary isolation, growth of additional strains were reported by several workers (Sheldon *et al,* 1945; Beveridge, 1946; Jennison *et al*, 1947; Dienst *et al,* 1948; Thomison, 1951; Goldberg *et al*, 1953a). The secondary cultivation of the organism in laboratory culture media consisting of egg yolk products had been attempted and reported (Dienst *et al*, 1948; Dunham and Rake, 1948; Dulaney *et al*, 1948; Goldberg *et al*, 1953a; Goldberg, 1962). How successful these culture methods were, could be not be evaluated, since none of the original cultured strains are available from any type culture collections.

2.2 PATHOGENESIS

Pathogenic micro-organisms adapt to infect a host by highly specialised mechanisms. Virulence factors are diverse as well as specific for micro-organisms and their hosts. For a successful pathogen, the ability to attach to host cells is a prerequisite, as is its ability to evade the host's immune response. Success of the pathogen depends on its ability to pass defence barriers and to reach its specific niche within the host. Some bacteria do so by living an intracellular life. To maintain the species, successful transmission to a new susceptible host is important (Finlay and Falklow, 1989).

Microbial pathogenicity has been defined as the "biochemical mechanisms" whereby micro-organisms cause disease. Not all pathogens have an equal ability to cause disease and the successful persistence or multiplication of a pathogen on or within a host is termed an infection, whilst an infection causing significant damage to the host is the disease. Generally, various protective mechanisms prevent microbial entry into an animal host. Pathogenic micro-organisms capitalise on damaged sites in the host barrier as points of entry. One such host barrier is the skin which can be damaged by trauma of various types, allowing organisms with pathogenic potential to enter. Relatively little is known about the microbial factors which ensure effective transmission from host to host. The sexually transmitted pathogens are transmitted by direct inoculation onto a new surface. This microbial strategy for survival avoids life in an external environment.

Microbial adherence requires receptors on the eukaryotic cell surfaces, which are usually specific carbohydrate residues and bacterial adhesins which are protein structures on the bacterial cell surface. Fimbriae (pili) are important adhesin carriers, whilst other non-fimbrial adhesins such as haemagglutinins mediate adherence to host cells (Finlay and Falklow, 1989). For most bacteria the chemotaxis of micro-organisms towards host surfaces might be important in the pathogenesis of the disease, however its role remains poorly characterised.

To pursue an intracellular lifestyle an organism must first penetrate the eukaryotic cell surface barrier and invade into the host cell and avoid the host defence immune system

(Moulder, 1985). The intracellular localisation places the organism in an environment potentially rich in nutrients, devoid of competing organisms and hidden from host defence. Within the host cell, the organism must be able to survive, multiply, and ultimately escape from the host cell to invade other host cells (Roszak and Colwell, 1987).

The initial recognition of the presence of intracellular Donovan bodies was considered to be a major breakthrough in the understanding of the disease, granuloma inguinale. There seems little doubt that these Donovan bodies are associated with granuloma inguinale, the presence of which are confirmed by the examination of Giemsa stained tissue smears, thus *C. granulomatis* seems to be at least a facultative intracellular pathogen. The histological examination of biopsied tissue shows a massive mixed inflammatory infiltrate and granuloma formation of the characteristic "granulomatous" lesions. The pathogenic mechanisms and the virulence factors of the aetiological agent *C. granulomatis* are not yet understood.

The lesions in granuloma inguinale develop (Figure 3) from an initial papule or vesicle. When allowed to progress untreated the initial lesion gradually becomes excoriated resulting in an elevated ulcer which spreads by continuity or contact autoinoculation to the surrounding tissue. The ulceration which follows, is typically granulomatous. Secondary inoculation to the surrounding sites plays an important role for spread and often initiates extensive inflammation of the cutaneous and subcutaneous tissues with necrosis and resultant scarring of the genitalia.

Characteristically, the lesions vary in size, are relatively painless, velvety and bright red in colour. They bleed easily and induration is variable. The initial genital lesions may be followed by "daughter lesions" which are largely due to autoinoculation (Dienst *et al,* 1949). These daughter lesions may coalesce to form extensive ulcerations. Metastatic haematogenous spread to bones, joints and liver occurs rarely (Packer *et al*, 1948; Rajam and Rangiah, 1954; Rajam *et al*, 1954). Occasionally the lymphatics may be involved with enlargement or suppuration of these glands (Chen *et al*, 1949; Freinkel, 1988). While ulceration in the inguinal area is common, lymph node enlargement is infrequent. The surrounding tissue is affected, producing a periadenitis and the formation of an

DISEASE DEVELOPMENT IN GRANULOMA INGUINALE

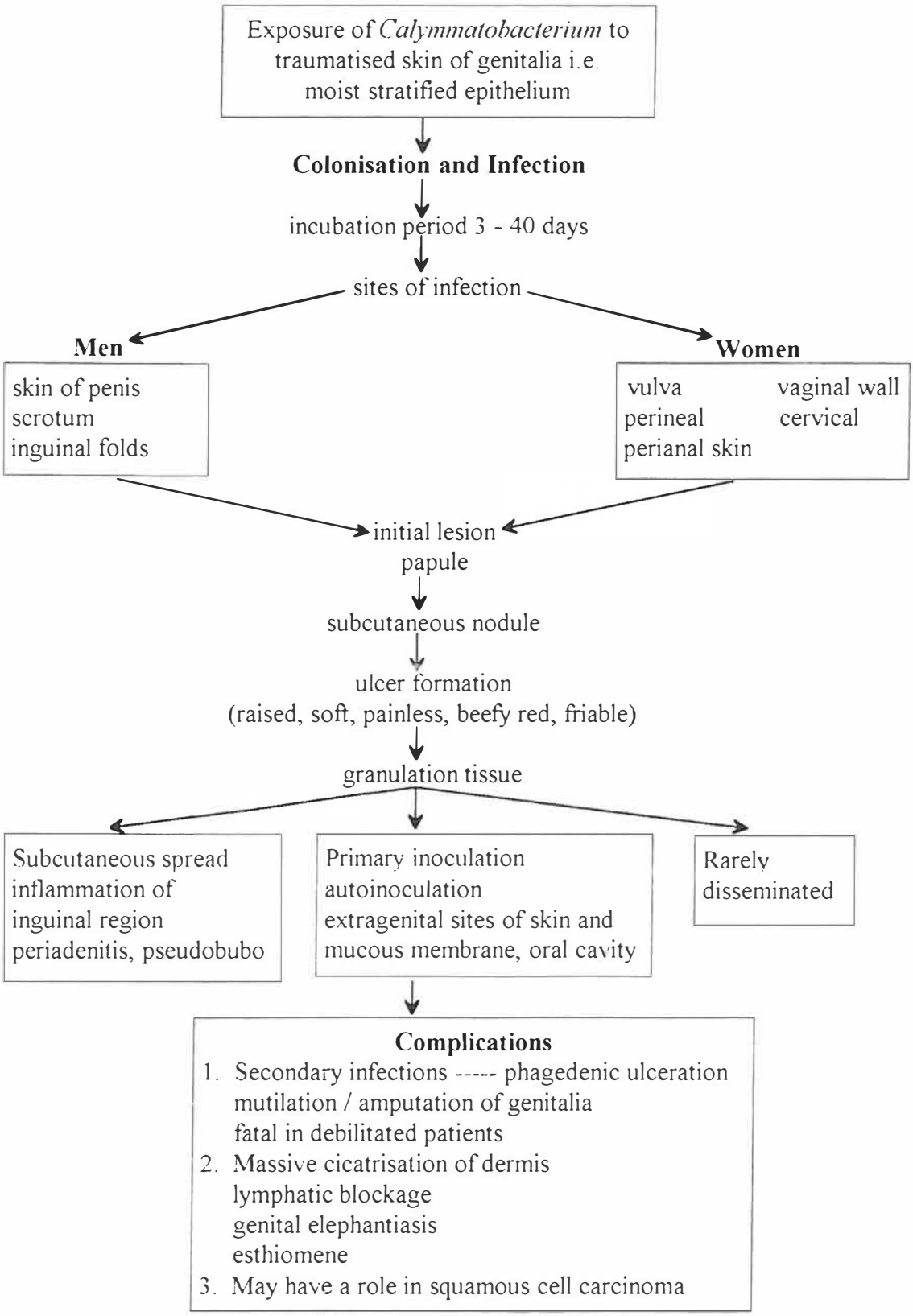


Figure 3: Diagram showing the development and progression of disease in granuloma inguinale.

abscess or subcutaneous "granuloma" termed "pseudobubo" (Greenblatt *et al*, 1939) which frequently ulcerate to form inguinal ulcers. The ulcer may heal with formation of a band - like scar around the genitalia with resultant lymphatic obstruction and pseudoelephantiasis (Halty, 1933; Schoch and Alexander, 1939; Dienst *et al*, 1949; Kuberski, 1980a; Sehgal and Shyamprasad, 1986; Sehgal *et al*, 1987a, Sehgal and Sharma, 1990). The precise pathogenesis of pseudoelephantiasis is still obscure. The mechanical pressure of scar tissue over the lymphatics together with a continuous chronic inflammatory response may be responsible for the condition, and this may be influenced by its duration (Sehgal *et al*, 1987a).

Numerous investigators have attempted to show the capacity of *C. granulomatis* to induce infections. McIntosh (1926) was the first to achieve the successful transmission of the disease experimentally by the subcutaneous implantation of "granuloma" tissue to another patient. This inoculum not only contained Donovan bodies but possibly other micro-organisms. However, he reported that the Donovan bodies were the causative agent and demonstrated changes in agglutinins, serum proteins and skin sensitivity, suggesting the production of antibodies against Donovan bodies.

The production of typical lesions in human volunteers was first demonstrated by Greenblatt *et al* (1939). Pus from a pseudobubo (unulcerated lesion) containing Donovan bodies was used in an attempt to experimentally produce granuloma inguinale in human volunteers and animals. In three humans, the experimental lesions were reproduced, however the inoculum was non-infectious for experimental animals. Dienst *et al*, (1948) using a culture failed to induce any disease in human volunteers or experimental animals. This would suggest that the culture was unlikely to be that of *C. granulomatis*. McIntosh (1926) and Greenblatt *et al* (1939) attempted to fulfill Koch's postulates which require the organism to be observed in disease, isolated and grown in pure culture; give rise to disease when inoculated into a susceptible host and be recovered from the diseased host. Although the organism was transferred successfully to a secondary host producing the characteristic lesions, Kochs' postulates were not entirely fulfilled since the inoculum was not a pure laboratory culture but exudate from a pseudobubo. In addition the organism could not be recovered in culture from the inoculated individual. The failure for the recovery of the infecting organism by

culture could have resulted in the lack of detection of any other concomitant organisms which might have been present. Thus, the authors could have transmitted a mixed infection with more than one pathogen, which could have existed in a symbiotic relationship, therefore not fulfilling Koch's postulates. However, these postulates are a useful historical reference and as such it should be noted that they were not regarded as a rigid criterion by Koch himself (Evans, 1976b). Presently in view of the inability to grow many presumed human pathogens in the laboratory, these criteria should not be binding (Evans, 1976b).

DeMonbreun and Goodpasture (1931a) aspirated material from two unruptured lesions from a Negro man who also had other ulcerated lesions of granuloma inguinale. This material was rich in Donovan bodies and presumed free from contaminating micro-organisms. They reported the isolation of a Gram negative bacillus belonging to the aerogenes group with morphologic characteristics of Donovan bodies. A group of six volunteer human beings and *Macacus rhesus* monkeys were inoculated with these cultures. Granuloma inguinale could not be induced by subcutaneous implantation of such cultures in the volunteers. After repeated inoculations in *Macacus rhesus* monkeys, nodules were visible without any evidence of ulcerative lesions as seen in chronic human infections. Donovan bodies were demonstrated in smears from nodules which may have existed from the original tissue implant.

Calymmatobacterium granulomatis and *Klebsiella pneumoniae* have been reported to be antigenically related (Packer and Goldberg, 1951). Experimental human inoculations with strains of *Klebsiella pneumoniae* failed to produce the characteristic lesions of granuloma inguinale (Packer and Goldberg, 1951). It has been shown that hyaluronidase which is present in semen has a potentiating effect in the initiation of lesions. However, the addition of hyaluronidase to the infecting inoculum described above did not influence the subsequent course of the disease (Packer and Goldberg, 1951).

Extensive experimental inoculations to produce granuloma inguinale lesions in animal models by various methods and routes have persistently failed and therefore the organism has not been shown to be pathogenic for laboratory animals (Greenblatt *et al,* 1939; Anderson, 1943; Anderson *et al*, 1945a; Beveridge, 1945). Granuloma inguinale is thought

to be a disease of low grade contagiousness. Direct contacts, including sexual contacts, do not necessarily result in an infection (Dienst *et al*, 1949). It is also unclear whether repeated exposure is required to produce an infection.

The incubation period has always been regarded as an "unsolved mystery" (Clarke, 1947) and has remained so. The exact incubation period is not known, but appears to have wide variation, ranging from several days to months. Experimental infections have been reported to result in ulcers within 50 days (Greenblatt *et al,* 1939) and, cultured organisms when transplanted in a volunteer have yielded Donovan bodies from the site in 60 days (Dienst *et al*, 1947). The results of these experiments suggest that granuloma inguinale is infectious, supposedly having an incubation period of 50 to 60 days. From the "patients" sexual history the reported incubation periods vary considerably ranging from as little as two days to as long as years (McIntosh, 1926; Clark, 1947; Sehgal and Shyamprasad, 1984). Sehgal and Shyamprasad (1984) have reported an average incubation period of 17,5 days with a wide range of 3 to 90 days, whilst McIntosh (1926) had determined it to be 42 days.

To elucidate the probable role of the immune response in the pathogenesis of granuloma inguinale, Sehgal *et al* (1987; 1991) estimated and characterised the circulating lymphocyte subpopulations in patients with granuloma inguinale. Although the CD4 to CD8 ratios were maintained in these patients, the absolute counts of the CD4 and CD8 cells were raised in all patients with ulcero-granulomatous type of lesions, whilst in patients with hypertrophic type lesions, the absolute number of B lymphocytes was raised, with a corresponding elevation in the concentrations of serum IgG and IgM (Sehgal *et al,* 1987; 1991a). Thus suggesting a TH1/TH2 differentiation of the immune response. It may be possible that these differing cell populations could be participating in determining the clinical expression of the disease. At the tissue level the ratio of CD4 to CD8 cells showed a highly significant change in patients with hypertrophic as compared to that in ulcerogranulomatous lesions. These findings suggests an important role of cell mediated immunity (Sehgal *et al,* 1991b). The CD4 cells have the ability to activate macrophages to extricate the organism. This association is demonstrated by the large number of histiocytes containing greater number of Donovan bodies in the hypertophic variant as compared to the ulcero-

granulomatous type of lesions (Sehgal *et al,* 1984).

2.3 EPIDEMIOLOGY

There has been much controversy regarding the transmission of granuloma inguinale. It has been generally considered to be sexually transmitted. Granuloma inguinale is one of the "rarer" forms of genital ulcer disease and is not a prominent disease in the western world though, it remains a significant cause of morbidity in specific areas of certain developing countries. The disease has occurred worldwide, but presently very few cases are reported from developed countries. For these countries, infections are usually associated with a history of travel and as a result of contracting the disease from areas where granuloma inguinale is endemic. Presently, the disease appears to have a specific geographical distribution, occurring predominantly in the tropical and subtropical regions with a concentration in certain regions and apparent absence in others.

Although epidemics of granuloma inguinale are uncommon, the largest epidemic has been recorded in South America and Papua New Guinea (Richens, 1985). In Dutch South New Guinea, between 1922 and 1952 an estimated 10 000 of a population of less than 15 000 were affected (Vogel and Richens, 1989). This high incidence was attributed to the widespread uninhibited sexual practices of tribe members. To date the condition is still prevalent on these islands.

The disease continues to be endemic in Papua New Guinea (Maddocks *et al*, 1976), in India (Anandam, 1979), especially in the south - east (Nair and Pandalai, 1934) where it was first described (McLeod, 1882;), the Caribbean and neighbouring parts of South America, the Guianas, Brazil (Jardim, *et al* 1990; Benzaken and Sardinha, 1995) mainly the north eastern regions and amongst the aboriginal community in the tropical zones of Australia (Cleland and Hickinbotham, 1909; Watsford and Alderman, 1953; Ashdown and Kilvert, 1979; Mitchell *et al,* 1986, Merianos *et al,* 1994). Additional foci are found in Vietnam (Shapiro and Breschi, 1974; Breschi *et al*, 1975) and Zambia (Bhagwandeen and Mottiar, 1972). Occasional case reports continue to appear from non endemic areas (Gould and Clark, 1966; Fujiwara *et al*, 1987; Hacker *et al,* 1992; Niemel *et al*, 1992), however it was felt

that these infections are generally acquired in endemic areas. Poor socioeconomic conditions have been one of the major contributory factors which have resulted in the endemicity of the disease. It is generally associated with third world conditions such as poverty, unhygienic conditions, overcrowding, general promiscuity amongst population groups and inadequate health care facilities (Rajam and Rangiah, 1954).

In South Africa, a significant focus has recently been identified in the Durban , Kwa Zulu Natal region (Hoosen *et al,* 1990, O'Farrell *et al*, 1990; 1991a; 1991b;1992; Bassa *et al,* 1993; Hoosen *et al*, 1996), although it had been reported previously from other areas (Freinkel and Counihan, 1983, Wistrand and Wegerhoff, 1985). A summary of studies on the occurrence of granuloma inguinale in South Africa and its neighbouring states is outlined in tables II and III respectively.

In the United States of America the disease was reported extensively (McIntosh, 1926; Fox, 1926; Marshak *et al*, 1948; Marmell and Santora, 1950) especially in the southern states, but the annual incidence has steadily declined and is now thought to be non existent (Greenblatt, 1947). Improved hygiene, better physical conditions and exposure to antibiotics used for conditions other than granuloma inguinale may have contributed to this decline.

The exact mode of transmission of the disease is unclear and the role of non - sexual transmission has been controversial. Evidence against the disease being sexually transmitted is that it is considered to be mildly contagious, requiring repeated exposure, with a relatively long incubation period for the development of most clinical cases. The rates of disease amongst regular sexual partners of patients with active lesions varies from 1% in the USA (Packer and Goldberg, 1950) and Papua New Guinea (Maddocks *et al*, 1976) to 12% to 52% in India (Lal and Nicholas, 1970). Nair and Pandalai (1934) were not convinced that the disease is sexually transmitted since in their series of 51 married patients, only three women gave a history of their stable marital partners having some kind of genital lesions, whilst the partners of the rest were free from disease. There is also a preponderance of the disease in women (Rajam and Rangiah, 1954; Greenblatt, 1947) and

TABLE II: OCCURRENCE OF GRANULOMA INGUINALE IN SOUTH AFRICA

Reference	Summary
Ricono, 1916	Described 6 cases in the Mount Fletcher area of the former Transkei. One patient claimed to have contracted the disease whilst working in the mines in Johannesburg
Pijper, 1918	Described 1 case in Johannesburg
Fraser, 1925	Described 18 cases among the coloured population of Cape Town. Mentions Donovan bodies but does not indicate whether they were seen in each case. Penis was destroyed in 4 cases
Manson-Bahr and Anderson, 1927	Reported 5 cases at the Seamen's Hospital, Royal Albert Dock, London, one of whom contracted the disease in Cape Town in 1922
Pfeiffer, 1939	Described granuloma inguinale in a Cape coloured female, a permanent resident in the Orange Free State. The patient presented with extensive ulceration of the genitalia
Duncan *et al*, 1981	Noted 1 case among 102 cases of sexually acquired genital ulcerations amongst black miners in Johannesburg
Freinkel and Counihan, 1983	Diagnosed eight cases on histological examination on the East Rand between July 1980 - April 1982
Freinkel, 1984	Reported 46 cases diagnosed on histological examination; most from the former eastern Transvaal (now Mpumalanga)
De Boer *et al*, 1984	Described 2 cases in Pretoria in women with vulval granuloma inguinale diagnosed on Papanicolaou stain
Coovadia *et al*, 1985b	Described a case with a sinus of the neck resembling actinomycosis; a vulval ulcer also present. The patient was from Durban
Wistrand and Wegerhoff, 1985	Described 30 cases between 1 September 1975 - 30 November 1977 and a further 13 cases 1 June 1981 - 15 September 1983 in the former eastern Transvaal (now Mpumalanga)
Leiman *et al*, 1986	Described 2 cases in Johannesburg in women with cervical granuloma inguinale diagnosed on Papanicolaou stain
Faber, 1987	Reported a single case in Bloemfontein in a patient who had never been out of the Orange Free State

Freinkel, 1988	Reported 2 patients from Johannesburg presenting with enlarged lymph nodes in the neck resembling tuberculosis, but biopsy showed granuloma inguinale
Hoosen *et al*, 1990	Detected 18 women from Durban with cervical granuloma inguinale, 50% of whom had a clinical diagnosis of carcinoma of cervix on admission
O'Farrell *et al*, 1990	Reported from the sexually transmitted diseases clinic, Durban on 23 of 55 men and 8 women with genital ulceration who were smear positive for Donovan bodies by the RapiDiff and May-Grunewald-Giemsa staining methods
O'Farrell *et al*, 1991a	Found 11 men with granuloma inguinale from 100 sexually acquired genital ulcerations attending a sexually transmitted diseases clinic in Durban
O'Farrell *et al*, 1991b	Found 16 women with granuloma inguinale from 100 sexually acquired genital ulcerations attending a sexually transmitted diseases clinic in Durban
O'Farrell, 1992	Reported 313 cases attending a sexually transmitted diseases clinic in Durban in 1988; Donovan bodies were detected in tissue smears stained by RapiDiff in 169 (129 men, 40 women) and histological diagnosis was made in two; 142 (126 men, 16 women) were diagnosed clinically
Bassa *et al*, 1993	Reported 61 women (26 pregnant; 35 non pregnant) with granuloma inguinale at a tertiary hospital (KEH), Durban, over a 3 year period
Hoosen *et al*, 1996	Reported 123 women with granuloma inguinale at a tertiary hospital (KEH), Durban, over a 3 year period. Diagnosis was made by tissue smear and / or histology. 42% were pregnant and 16% had antibody to HIV

TABLE III: OCCURRENCE OF GRANULOMA INGUINALE IN THE NEIGHBOURING STATES OF SOUTH AFRICA

Reference	Summary
Willcox, 1950	Reported on 17 cases of granuloma inguinale in Southern Rhodesia (now Zimbabwe); 11 males and 6 females
Brain, 1951	Described a Shangaan male in southern Rhodesia (now Zimbabwe) with granuloma inguinale with extensive oral involvement
Kingsley, 1967	Described a young African male with granuloma inguinale in Bulawayo, Rhodesia (now Zimbabwe)
Bhagwandeen and Mottiar, 1972	Reported 6 cases of granuloma inguinale (venereum) in African females, with 1 case involving the cervix and simulating carcinoma
Bhagwandeen and Naik, 1977	Described 40 patients (5 males; 35 females) with histologically proven granuloma venereum (granuloma inguinale) in Zambia
Veen, 1979	Described a single case of granuloma inguinale in a girl in Botswana

being an infectious disease which is sexually transmitted, then both men and women should be equally affected. In prostitutes the disease is not as prevalent as other sexually transmitted diseases (Marmell and Santora, 1950). In addition there is a high prevalence of extra-genital infections without a genital focus (Sehgal and Shyamprasad, 1986). Goldberg (1962) reported the isolation of the causative agent from the faeces of a patient with cervical granuloma inguinale and postulated that the rectum was the reservoir for the organism and the vagina lying in close proximity to the rectum becomes infected either by autoinoculation from the rectum or the clinical disease occurs after sexual or non sexual trauma of infected sites.

To support the role of sexual transmission, there has always been a history of sexual contact, the commonest site of the disease is the genitalia, the age of peak incidence corresponds to the period of maximum sexual activity and the disease often occurs with other sexually transmitted diseases. The sex distribution of the disease is inconsistent and varies from site to site mainly depending on the facility and whom it is servicing. There is overwhelming evidence that granuloma inguinale frequently occurs concomitantly with other sexually transmitted diseases. In particular it has been found together with syphilis (Lal and Nicholas, 1970; Anandam, 1979; Vacca and MacMillan, 1982; Sehgal and Shyamprasad, 1984; Bassa *et al*,1993, Hoosen *et al*, 1996), lymphogranuloma venereum (Vacca and MacMillan, 1982) and warts (Anandam, 1979). These studies provide significant evidence associating granuloma inguinale with sexual activity and therefore consider it to be sexually transmitted.

The disease has been recognised in prepubertal children. Zigas (1971) screened a New Guinea population and detected lesions in 4,4% of children 1- 4 years of age and in 4.9% of persons over 15 years, whilst no disease was detected in the age group 5 - 14 years. He suggested that the mode of transmission and infection to young children was probably by sitting on laps of diseased patients or relatives, whilst adult infections were associated with sexual intercourse (Zigas, 1971). The role of child abuse amongst this group of infected children could not be excluded. The rectal and penile lesions have been associated with anal intercourse, especially with homosexual practices (Marmell, 1958; Goldberg, 1964).

Another factor influencing the endemicity of the disease in India is thought to be the climatic conditions. In areas where there is a high incidence, it appears to be associated with moderate humidity and constant high temperatures throughout the year (Rajam and Rangiah, 1954; Sehgal and Shyamprasad, 1986), but this has not been supported by any other studies.

There is an overwhelming preponderance of the disease in the Aryan and African ethnic groups as compared to Caucasians (Rajam and Rangiah, 1954; Greenblatt, 1947). It is thought that the stratum corneum of the genito-inguinal region is thinnest among the former, and the apocrine glands more abundantly distributed, and that these two factors in the composition of the skin account for the greater predisposition and racial susceptibility (Rajam and Rangiah, 1954). In the USA the disease was seen exclusively amongst the Negro population and occurred relatively infrequently amongst the fairer skinned. Those Caucasians that were affected were considered to be "poor whites". In addition the disease runs a milder course in the Caucasian than in the Negro (Greenblatt, 1947).

Although there is an important association between histocompatibility antigens (HLA) and certain diseases, its potential importance in sexually transmitted diseases has not been fully explored. Typing of HLA is unreliable in confirming or refuting any diagnosis in an individual case, since it lacks sensitivity and specificity. However, it is useful in identifying individuals at risk for the development of certain diseases (Bird, 1991), especially in some patients who have repeated episodes of certain types of sexually transmitted diseases (Kuberski, 1980b). Although there is evidence that poor socioeconomic conditions greatly influence the development of infections, the apparent racial selectivity suggests the possibility of intrinsic susceptibility factors. To assess the role of these factors, the finding of HLA B57 in 15% and HLA A23 in 5% of patients with granuloma inguinale and HLA B57 in 5% and HLA A23 in 19% of normal controls, although not statistically significant, suggests a possible link between HLA B57 and granuloma inguinale and a trend towards resistance to disease with HLA A23. The coexistence of these alleles or immune response genes could result in linkage disequilibrium, altering disease susceptibility (O'Farrell and Hammond, 1991). Further studies are required to assess the role of genetic factors.

2.4 DIAGNOSIS

2.4.1 CLINICAL DIAGNOSIS

The clinical features of granuloma inguinale lesions have been described extensively. These are usually characteristic enough to suggest a correct diagnosis in most cases (Richens, 1991), however, because of the different sites of infection and multiple morphologies, a clinical diagnosis of granuloma inguinale may be difficult.

As early as 1882, McLeod described the typical lesions of granuloma inguinale, but the description by Rajam and Rangiah (1954) has remained the most accurate and has been confirmed by many workers (Nair and Pandalai, 1934; Lal and Nicholas, 1970; Anandam, 1979; Sehgal and Shyamprasad, 1984; O'Farrell *et al*, 1994).

In most cases the lesions develop as a papule or subcutaneous nodule which may be preceded and accompanied by pruritis. The lesion at first is firm and later enlarges and softens with multiple nodules appearing. Nodules become larger and eventually ulcerate revealing a beefy granulating ulcer (Figure 4) which is relatively painless and bleeds easily on touch. Smaller satellite papules may ulcerate and coalesce with the initial lesion. The pathological classification of clinical lesions was originally described by Halty (1933), D'Aunoy and Von Haam (1938) and Packer and Dulaney (1949). Sehgal and Shyamprasad (1984) have clearly delineated the morphologic variants. These being the ulcerative or ulcero-granulomatous form - which is the fleshy, exuberant lesion presenting as a beefy red granulomatous ulcer, usually single, non tender, non indurated which bleeds profusely on touch; the hypertrophic or verrucous form - which consists of ulcerating growth with a raised, irregular edge or surface, drier than the ulcerative variety, with an elevated granulomatous base; the necrotic type - which gives rise to extensive destruction of genitalia with profuse, foul smelling exudate; and the sclerotic variety - which presents as a band - like scar in and around the genitalia.

The inaccuracy of a clinical diagnosis of genital ulcer disease is now well recognised (Dangor *et al*, 1990; O'Farrell, *et al*, 1994). Granuloma inguinale may be confused with

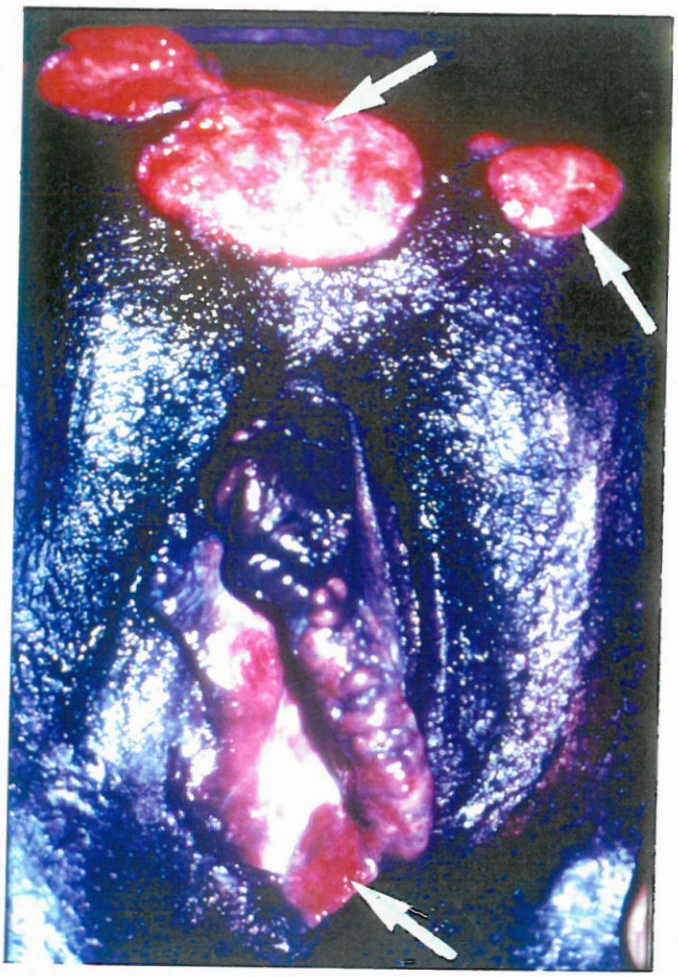


Figure 4: Lesions of granuloma inguinale. Note elevated, soft, friable, granulomatous, beefy red lesions in a female patient who presented to the antenatal clinic of King Edward VIII hospital, Durban.

primary and secondary syphilis (Birch, 1929; Brandt and Gatewood, 1941) and multiple chancroidal lesions (Giglioli, 1930; Kraus *et al*, 1982; Werman *et al*, 1983; Verdich, 1984). The clinical diagnosis is made more difficult in the early stages of the infection (Ross and Kaupp, 1927) by the presence of mixed infections (Vacca and MacMillan, 1982), atypical ulceration (Sehgal *et al,* 1979; Spagnola *et al*,1984), unusual manifestations (Harris, 1930; Crane and Kimball, 1940; Lyford *et al*, 1944; Hill *et al*, 1949; Jannach, 1958; Bhaskar *et al*, 1965; Garg *et al*, 1978; Jofre *et al*,1976; Graney and Bodon, 1976; Murugan *et al*, 1982; Leung and Mc Cartney, 1990) and necrotic lesions which are secondarily infected especially with fusospirochaetes (Packer and Dulaney, 1949).

In the literature, granuloma inguinale has often been confused with lymphogranuloma venereum (LGV). The term "lymphogranuloma inguinale" has been used by some authors to refer to granuloma inguinale and by others to refer to LGV (Kolmer, 1941). The similarities of the clinical picture and geographical distribution has resulted in additional confusion. The diagnostic features of secondary LGV like labial fenestration and the combination of ulceration with elephantiasis are also a common finding in patients with granuloma inguinale (Richens, 1991).

2.4.1.1 Sites of infections

2.4.1.1.1 *Genital infections*

The predominant site for primary lesions is the genital region (Rajam and Rangiah, 1954). In males, the commonest location is on the prepuce, or the coronal sulcus with extension to the genital crural or inguinal folds (Sehgal and Shyamprasad, 1984). In homosexuals granuloma inguinale is confined to the perianal and anal region (Rajam and Rangiah, 1954; Marmell, 1958; Goldberg and Bernstein, 1964). In females, it presents most commonly on the labia or fourchette and may spread to the perineal and perianal skin (Sehgal and Shyamprasad, 1984). Many other sites may be involved either as a primary event or by secondary spread.

2.4.1.1.2 *Ascending genital infections*

In general, if granuloma inguinale is appropriately treated, it tends to be benign with no known sequelae. The course of untreated granuloma inguinale is chronic with continuity and contiguity, with no tendency to spontaneous healing and cure. The prognosis is generally considered to be unfavourable without appropriate treatment. Most patients tend to seek medical care after symptoms have persisted for several months probably because of the fact that the lesions are painless and most patients are from disadvantaged low socioeconomic population groups where access to health care may be problematic. As a result, the disease is progressive with extensive local tissue destruction occurring for several months or years resulting in mutilation or autoamputation of the external genitalia (Fritz *et al*, 1975) as shown in figure 5. Extensive ulceration with elephantiasis - like enlargement of the labia, vulva (Schoch and Alexander, 1939) or penis and scrotum (Sehgal *et al*, 1987a; Sehgal and Sharma, 1990) is found in approximatly 5% of patients with granuloma inguinale. Figure 6 shows swelling of the labia majora resulting in elephantiasis (pseudoelephantiasis) in a patient with a history of lesions of 6 months duration who presented to the antenatal clinic of King Edward VIII hospital, Durban. The mechanical pressure of scar tissue over the local lymphatics coupled with a continuing chronic inflammatory response may be responsible for the condition, however, the precise pathogenesis of this is obscure (Sehgal *et al*, 1987).

The occurrence of the disease in the deeper parts of the vagina (Murugan *et al*, 1982) and cervix (Arnell and Potekin, 1940; Guerriero *et al*, 1942; Adams and Packer, 1955; Stewart, 1964; Sengupta and Das, 1984; Hoosen *et al*, 1990) has been observed either in association with vulval lesions or as primary lesions. The severity and extent of the primary lesions of the cervix can often simulate cancer with case fatality rates of 5,2% (Arnell and Potekin, 1940) and 14,3 % (Pund and McInnes, 1944). Primary cervical granuloma inguinale may be associated with involvement of the endometrium (Bhagwandeen and Mottiar, 1972; Scrimgeour *et al*, 1983) and the uterus (Kalstone, 1961), fallopian tubes and ovaries (Pund and Auerbach, 1944; Pund and Gotcher, 1948; Marmell *et al,* 1952). In males, infections of the buttocks (Jennison *et al*, 1947) and epididymis (Jannach, 1958) have been reported.

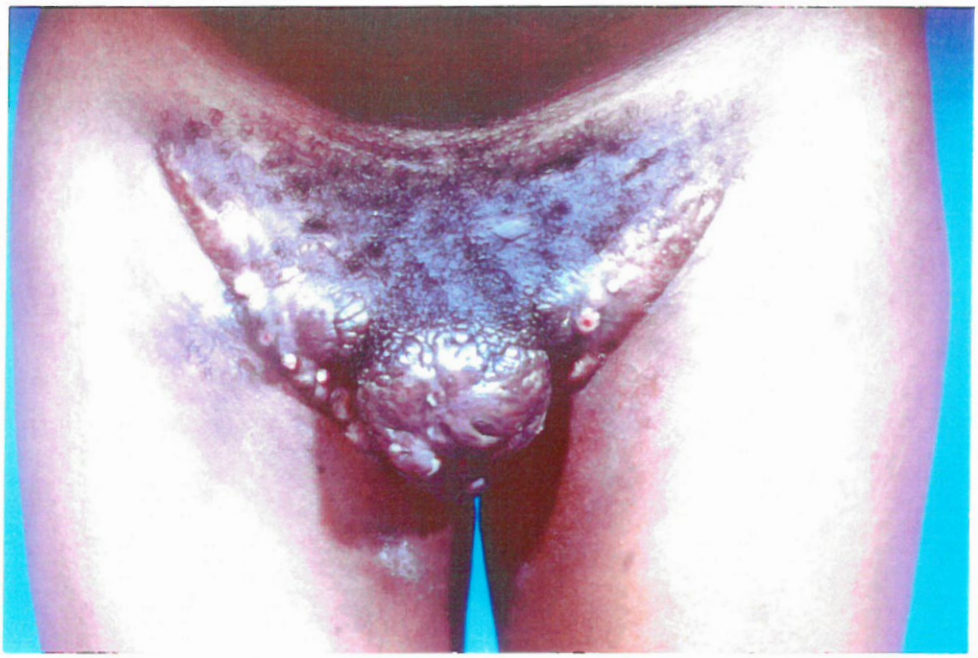


Figure 5: **Advanced lesions of granuloma inguinale showing severe mutilation of the external genitalia in a female patient who presented to the gynaecology outpatient clinic of King Edward VIII hospital, Durban with longstanding lesions.**

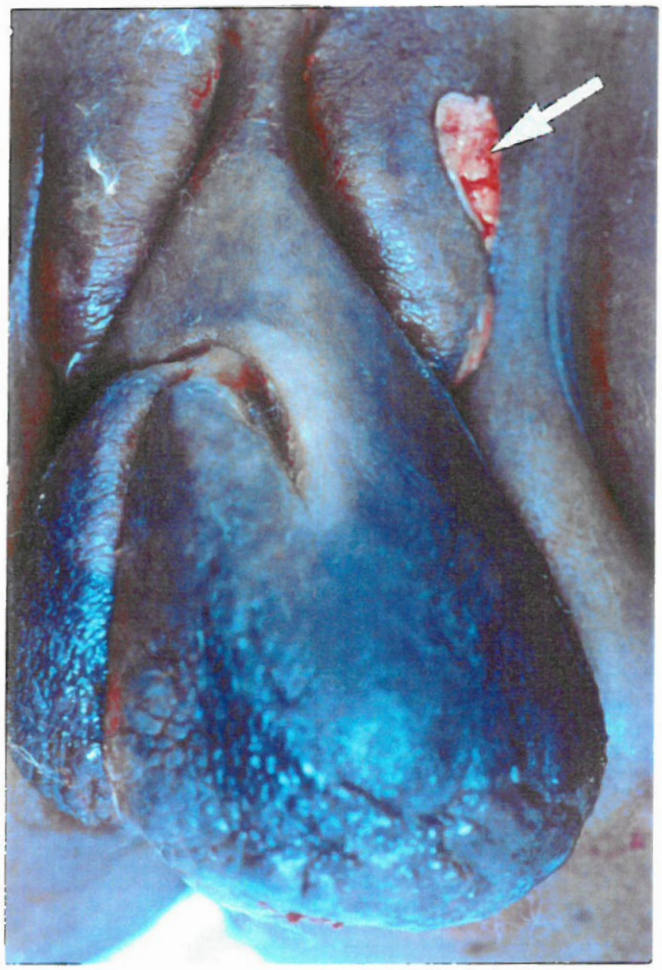


Figure 6: **Swelling of the labia minora resulting in elephantiasis (pseudoelephantiasis) in a patient who presented to the antenatal clinic of King Edward VIII hospital, Durban with longstanding granuloma inguinale.**

An association between granuloma inguinale and carcinoma of the genitalia has been suggested but not clearly established (Kuberski, 1980a). It may coexist (Beerman and Sonck, 1952) or follow chronic granulomatous disease of the vulva (Hay and Cole, 1976; Sengupta, 1980; 1981; or penis (Goldberg and Annamunthado, 1966). It has been postulated squamous cell carcinoma may develop especially in young patients with long standing granuloma inguinale that does not respond to specific therapy (Alexander and Shields, 1953; Rajam and Rangiah, 1953; Barnes *et al*, 1990). It is unclear whether chronic granulomatous infection caused by *C. granulomatis* enhances the possibility of development of cancer (Kuberski, 1980a).

2.4.1.1.3 *Extra genital infections*

Although the majority of cases of granuloma inguinale are confined to the genital region, primary and secondary lesions at numerous extragenital sites have been described (Bhaskar *et al*, 1965; Sehgal *et al*, 1979). Extragenital lesions usually follow or are found in association with perineal or genital lesions. The reported prevalence of extragenital lesions in patients with granuloma inguinale is approximately 6% (Greenblatt *et al*, 1939; Rajam and Rangiah, 1954; Cherny *et al*, 1957).

The most frequently reported extragenital lesions are those of the oral cavity (Donovan, 1905; Rajam and Rangiah, 1954; Hanna and Pratt-Thomas, 1948; Coovadia *et al*, 1985), lips (Hanna and Pratt-Thomas, 1948), above the mouth (Bhaskar *et al*, 1965) and skin (Sehgal *et al*, 1979). Other sites such as subcutaneous sinus tract (Garg *et al*, 1978), rectum and colon (Crane and Kimball, 1940) are less commonly affected.

Haematogenous dissemination has been reported with multiple system involvement (Rajam and Rangiah, 1954). There is a predilection for focal viscera or bone resulting in osteolytic lesions (Lyford *et al*, 1944; 1946; Cherny *et al*, 1957; Packer *et al*, 1948) which may radiologically resemble leukaemia or multiple myeloma (Kirkpatrick, 1970). In a patient with systemic granuloma inguinale Rajam and Rangiah (1954) reported the presence of osteolytic lesions, in addition to a growth of the cervix. Donovan bodies were observed in the lesions of the cervix, bone, bladder and inside liver cells. Other unusual sites of

involvement reported are the lung, liver and spleen (Lyford *et al*, 1946; Rajam *et al*, 1954).

In pregnancy the lesions of granuloma inguinale have a tendency to proliferate and recur, and delivery through an untreated infected cervix predisposes to haematogenous dissemination of the disease (Cherny *et al,* 1957) which may be fatal (Pund and McInnes, 1944). Neonates delivered through an infected cervix are exposed to untreated lesions and are also at risk of acquiring skin infections (Scott *et al*, 1952).

2.4.2 LABORATORY DIAGNOSIS

A limited number of laboratory techniques are available to aid the diagnosis of granuloma inguinale. Dienst *et al* in 1948 reviewed the diagnostic methods and suggested the demonstration of Donovan bodies in stained tissue preparations; detection of complement fixing antibodies in patients sera and a positive skin reaction to the intradermal inoculation of Donovan antigen as acceptable criteria. Sehgal and Shyamprasad in 1986 concluded that appropriately stained tissue specimens from active lesions still remain the most reliable diagnostic procedure (Sehgal and Shyamprasad, 1986).

2.4.2.1 Microscopic examination of tissue smears

The laboratory diagnosis of granuloma inguinale relies on the the direct visualisation of intramonocytic Donovan bodies in smears or biopsy specimen subjected to either crush preparation or thin sections (Marmell and Prigot, 1960). The finding of Donovan bodies provides a simple, highly specific way of confirming the diagnosis and can be demonstrated in 60% to 80% of cases (D'Aunoy and Von Haam, 1938), however, it lacks sensitivity. In patients with negative smears, the demonstration of Donovan bodies may only be possible after a careful examination of tissue sections. It is imperative that the selection of site for the collection of specimens, using good technique in making smears, thorough searching of such specimens, and familiarity with the tissue appearance of Donovan bodies are all important in assuring success (Dienst *et al*, 1948; Cannefax, 1948; Richens, 1991).

The technique for obtaining, preparing and staining for microscopic examination of tissue specimens for the demonstration of Donovan bodies has been described (Cannefax, 1948; Packer and Dulaney, 1949; Greenblatt and Barfield, 1952; Richens, 1991). An active area of ulceration should be selected and cleansed with saline soaked gauze to remove superficial granulation tissue, since the subsurface of such granulation tissue is rich in Donovan body content. Cells may be obtained by a variety of means. Direct impressions on a glass slide obtained from the ulcer base are not adequate because surface debris and secondary bacterial contaminants obscure the microscopic identification of Donovan bodies. Small pieces of the infected tissue can easily be detached using a forceps or scalpel blade or a cotton swab rolled over the surface of the lesion. If tissue is obtained, impression smears can be made on glass slides or crushed between two slides to deposit the tissue cells evenly on the surface of both slides (Cannefax, 1948). A biopsy specimen, preferably subjected to thin sections is recommended for lesions in which the organisms are likely to be scarce, where smear or crush specimens are likely to fail, or in very early or sclerotic and cicatricial type of lesions or lesions with heavy superinfection. The diagnosis is best made from tissue crush preparations of specimens stained with Wright's, Leishman, Giemsa stain or a special stain consisting of 1% pinacyanole in methyl alcohol (Greenblatt and Barfield, 1952). Although the Giemsa is time-consuming, the newer rapid Giemsa techniques (RapiDiff) are a useful adjunct (O'Farrell *et al*, 1990). When the Wright's stain is used, the initial staining in undiluted solution for one and a half minute has been recommended (Dienst *et al*, 1948). For formalin fixed embedded tissue, haematoxylin-eosin stained sections have been less useful for the detection of Donovan bodies (Davis, 1970; Dodson *et al*, 1974), whilst the use of Delafield's haematoxylin with a small amount of eosin was favoured by Pund and Greenblatt (1937). Although considerably longer to perform the use of Giemsa staining or silver staining techniques such as the Warthin-Starry has been advocated. The use of semi - thin sections, although more expensive and time consuming, has demonstrated Donovan bodies with impressive clarity (Davis, 1970; Jain *et al*, 1989) and has been recommended for difficult cases (Richens, 1991). Good results have been obtained with a slow Giemsa (Sehgal and Jain, 1987) and with thionine azure II basic fuchsin stains (Jain *et al*, 1989).

The morphological details of Donovan bodies are more easily recognised in well made

smears than in sections (Packer and Dulaney, 1949, Marmell and Santora, 1950). The organisms are present inside the cytoplasm of mononuclear cells (Figure 7). These host cells may exhibit vacuolation inside the cytoplasm. Characteristically two different forms of the Donovan body are recognised. The mature form is ovoid or bean shaped, varying from 1μm to 1,5μm in length and 0,5μm to 0,7μm in thickness, with a well defined pinkish material surrounding a bacillary body. The dark blue chromatin inclusions are arranged at the poles. The capsule may either stain bright pink or remain unstained. The morphology of the immature organism may be coccoid, diplococcoid or bacillary, the size which varies from 0,6μm to 1,0μm, with the diplococcoid form resembling a closed "safety pin" (Sehgal and Shyamprasad, 1986). These characteristic Donovan bodies have been visualised in inguinal pseudobubo aspirates (Cannefax, 1948), cytology specimens (De Boer *et al*, 1984; Leiman *et al*, 1986; Golfo and Galindo, 1990) and in an isolated case in peripheral blood monocytes (Packer *et al*, 1948).

2.4.2.2 Histological examination of tissue biopsy

Generally in the past, most pathologists reported on tissue biopsy specimens in a descriptive rather than a diagnostic manner. In sections from biopsy specimens of a chancre, the presence or absence of spirochaetes are reported accordingly, often resulting in a conclusive diagnosis of syphilis. In cases of granuloma inguinale no specific aetiological agent was ascertained and only the description of the dermal and epidermal changes were noted. These were often non specific. Galloway (1897) reported on the histology of patients with ulcerating granuloma of the pudenda which comprised leucocytes in small collections and cells which were larger than leucocytes with rounded nuclei of moderate size and protoplasm frequently containing "granules". No specific aetiological agent was ascribed to the disease in his report.

Since the end of the nineteenth century granuloma inguinale had been confused with carcinoma. It was generally assumed that it was a new growth or tumour (Conyers and Daniels 1896 cited by Rajam and Rangiah, 1954; Galloway, 1897) and numerous authors have reported cases of granuloma inguinale being either diagnosed as or simulating carcinoma (D'Aunoy and Von Haam, 1937; Pund and Greenblatt, 1937; Greenblatt, 1940;

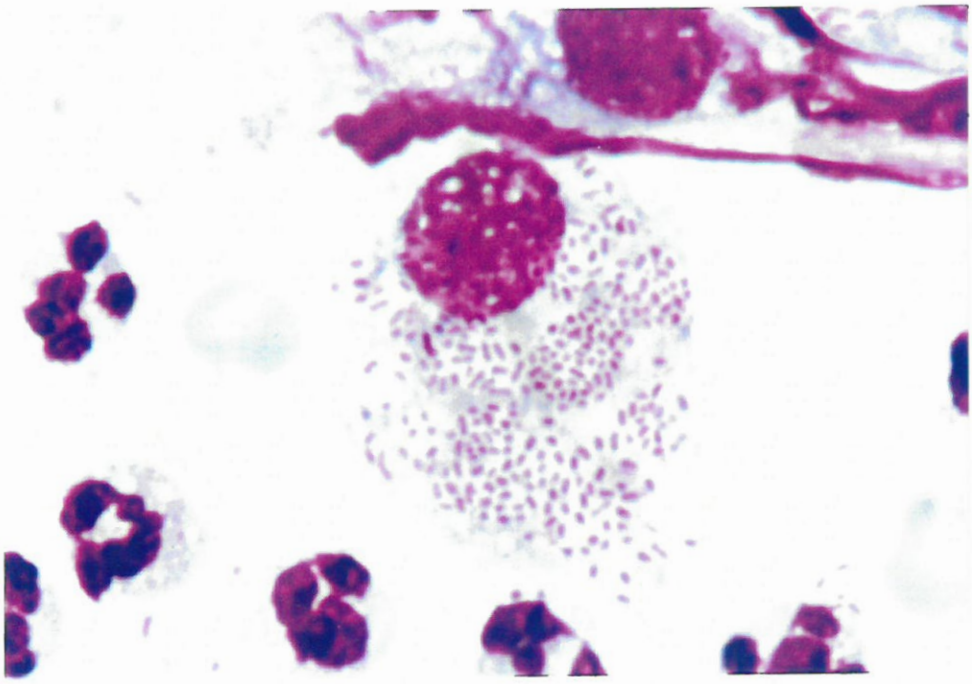


Figure 7: Photomicrograph showing RapiDiff stained preparation of granulation tissue with intracellular Donovan bodies in macrophages (Mag x500).

Rajam and Rangiah, 1954). Although granuloma inguinale and cancer may co-exist (Sengupta, 1981), the histological features are quite distinct (Greenblatt and Dienst 1948; Nayar *et al,* 1981) to differentiate the two. Pund and Greenblatt (1937) recognised that the histopathology was unique to make a diagnosis of granuloma inguinale. The cellular response was massive in which granulation tissue was surcharged with plasma cells, polymorphonuclear leucocytes which were either diffuse or as focal collections, whilst lymphocytes were rare. The pseudoepitheliomatous changes were variable (Phillpot, 1947). The pathognomonic large mononuclear cells with intra - cytoplasmic vacuoles containing deeply stained bodies were of paramount importance and the recognition of these cells were specific for the diagnosis of granuloma inguinale.

The distinctive histological features have been described by several other workers. The surface epithelial changes in granuloma inguinale were seen as ulcerations and erosion with occasional hyperkeratosis and thickening of the granular layer (Beerman and Sonck, 1952). The epidermal changes were variable with evidence of marked acanthosis and pseudoepitheliomatous hyperplasia of varying degree (Beerman and Sonck, 1952; Nayar *et al,* 1981; Sehgal *et al,* 1984). The polymorphonuclear leucocytes usually vary in number and are present as either diffuse sprinkling or as focal collections and result in the formation of microabscesses in a few cases (Pund and Greenblatt, 1937; Nayar *et al*, 1971; Sehgal *et al,* 1984) especially, in very active lesions (Pund and Greenblatt 1937). Scattered eosinophils may be present.

The dermal blood vessels were usually dilated and proliferation was prominent with significant endothelial changes. The dermal changes were primarily inflammatory consisting of dense infiltrate predominantly of plasma cells and mononuclear cells with varying numbers of histiocytes. A few large cells, 25μm to 90μm in size may contain the Donovan bodies either scattered or lying within the inner surface of the vacuole (Pund and Greenblatt, 1937; Nayar *et al*, 1981; Sehgal *et al*, 1984; Freinkel, 1987). Both intra and extracellular Donovan bodies with different morphologic features such as coccoid, coccobacillary or bacillary, as well as the mature capsulated forms are present. These are difficult to demonstrate with haematoxylin eosin staining, but are easily identifiable on Giemsa stained smears. In tissue sections, special stains such as Delafields haematoxylin

and silver stains such as Dieterles (Greenblatt and Barfield, 1952) and the Warthin-Starry stain (Freinkel, 1987) have been used to demonstrate Donovan bodies with remarkable clarity. With the silver stains, the organisms stain black, whereas with Giemsa they are seen as bluish red bodies. To increase the specificity of the diagnosis of granuloma inguinale, Donovan bodies have been identified in tissue sections using an immunoperoxidase method (Africa, 1990).

In granuloma inguinale, the histological picture is classical consisting of a mixed inflammatory infiltrate of plasma cells, neutrophils and histiocytes containing Donovan bodies with a conspicuous absence of lymphocytes.

2.4.2.3 Electron microscopic examination of tissue biopsy

Electron microscopic studies revealed discontinuous and swollen endothelium in the lesion and the organisms located within the phagosome of the histiocyte, but rarely seen in neutrophils or free in the extracellular space (Dodson *et al*, 1974; Chandra *et al*, 1989).

The ultrastructural features of *C. granulomatis* consisted of a corrugated cell wall with villiform protrusions, an outer trilaminar membrane and an extracellular clear zone presumably the capsular material (Kuberski *et al*, 1980). The cytoplasm contained electron dense homogenous inclusions, usually found at the periphery of the cell body.

As with most bacteria, the electron microscopic appearance of *C. granulomatis* is not specific enough to allow definite diagnosis of granuloma inguinale or identification of *C. granulomatis* (Davis, 1970; Dodson *et al*, 1974; Kuberski *et al*, 1980).

2.4.2.4 Serological diagnosis

The serological diagnosis of a disease depends on the availability of specific antigen for the detection of a specific immune response. The specific antigen is usually any component of the bacterium that will elicit a specific response. The inability to cultivate *C. granulomatis in vitro* has made it impossible to isolate and study the individual antigens by

conventional techniques. Furthermore, recombinant DNA techniques have not been utilised to provide an unlimited source of well defined *C. granulomatis* antigen, enabling the study of individual antigenic components for the optimal serological diagnosis of granuloma inguinale. Presently, serological tests for the diagnosis of granuloma inguinale are non existent.

A number of antibody tests have been used to determine the immune response, however, whole cell protein extracts have been used as antigen preparations to measure such antibodies and therefore the tests have lacked the use of any specific antigenic determinants / epitopes off the organism. McIntosh (1926) has postulated that the antibody production against Donovan micro-organisms was measured on the basis of agglutinins, precipitin and skin reactions. Anderson *et al* (1945b) prepared an antigen consisting of the mucinous substance of the embryonic yolk culture, which they assumed to be of capsular origin. Although the skin reactions with this antigen were mild they were positive in six cases of granuloma inguinale. The capsular material used by them was found to elicit precipitation reaction in 18 out of 19 cases, and complement fixation tests were positive in 12 out of 15 cases of granuloma inguinale. The antigen prepared from encapsulated organisms elicited a definite skin reaction (Kornblith, 1944; Chen *et al*, 1949) in patients with granuloma inguinale, whilst, the antigen prepared from unencapsulated organisms did not (Dulaney *et al*, 1948). These tests established the definite immunologic relationship of *C. granulomatis* to granuloma inguinale. In addition it suggested that *C. granulomatis* possessed antigenic properties and that the antigen specificity was most likely due to the capsule. However, since the capsular antigens were not further characterised, the specific epitopes have not been identified.

The growth of the organism in yolk sac by Anderson *et al* (1945a) prompted their efforts to develop a suitable antigen for complement fixation. They attempted to sensitise roosters by repeated intravenous injections of infected embryonic yolk and demonstrated the presence of antibodies by the positive ring flocculation, sedimentation tests as well as skin reaction with the capsular substance which was obtained by chemical treatment of the embryonic yolk. In a propective study Anderson demonstrated the presence of specific humoral antibodies in sera of 18 of the 19 patients' with granuloma inguinale by *in vitro*

precipitation and complement fixation tests (Anderson *et al* 1945b).

Due to the lack of availability of culture as a source of antigen, tissue extracts or pus containing Donovan bodies has been used as antigen. Pandalai and Nair (1934) prepared antigen of tissue scrapings from the edge of ulcers. This antigen was injected into the forearm of patients with granuloma inguinale resulting in an allergic sensitisation reaction. Kornblith (1944), using tissue extracts as antigen undertook a specific intradermal test and described the characteristic microscopic pathology to that of an epitheloid reaction which is generally common with most chronic granulomatous disease. An antigen prepared from a metastatic abscess containing Donovan bodies yielded serological evidence of granuloma inguinale when used in complement fixation tests. Twenty one of 25 patients with established granuloma inguinale gave positive reactions (Dulaney and Packer, 1948).

Dulaney *et al*, (1948) undertook complement fixation tests with antigens prepared from a strain of *Donovania granulomatis* grown on a coagulated egg yolk Locke's solution medium and found that 22 of the 24 sera of granuloma ingunale patients reacted positively. Goldberg *et al*, (1953b), prepared three antigens from two cultured strains (Anderson and Georgia strain). Sera from 136 of 151 patients with granuloma inguinale fixed complement with one or more antigens, whilst with one exception, 112 controls failed to respond positively. The authors reported that the strength of the test was proportional to the duration of the disease. On the basis of serology Goldberg *et al*, (1966) found that nine of their 62 cases of squamous cell carcinoma reacted positively with Donovania antigen.

Maddocks *et al*, (1976) using complement fixation tests with antigen from Anderson strain reported the presence of antibodies in all but one of the 23 cases of granuloma inguinale and in nine out of fourteen cases of clinically positive but smear negative patients, indicating a role for serology in the diagnosis of granuloma inguinale. The complement fixation tests with *Klebsiella pneumoniae* antigen was found to be sensitive for granuloma inuinale, but less sensitive than the test using *Donovania* antigen.

Recently, Freinkel *et al*, (1992) reported to have developed a serological test using an indirect immunofluorescence method. The antigen used was the organism present in tissue

sections, diagnosed as granuloma inguinale. The test was found to have a sensitivity of 100% and a specificity of 98% with a positive predictive value of 89% and a negative predictive value of 100%.

2.5 AETIOLOGY

The aetiology of granuloma inguinale had been obscure for a number of years. Donovan in 1905, described the presence of intracellular bodies in the exudate of an oral lesion of from a patient who had also suffered from genital lesions. He regarded them to be protozoal in nature and described them as "gigantic bacilli with rounded ends". Carter (1910) described them as "bean shaped bodies resembling the gregarine form stage of a herpetomonas or crithidia", whilst some thought them to be encapsulated intracellular diplococci aetiologically related to the organism of rhinoscleroma (*Klebsiella rhinoscleromatis*). In 1913 Aragao and Vianna named them *Calymmatobacterium granulomatis.* When Anderson *et al* (1945a) isolated the organism by yolk sac inoculation and suggested the name as *Donovania granulomatis.* The name *Donovania* did not have priority over *Calymmatobacterium* (Dienst and Brownell, 1984) and the latter name has persisted. Since then a number of workers have confirmed Donovans findings and *Calymmatobacterium granulomatis* has been clinically proven to be the causal agent of granuloma inguinale (Dienst *et al,* 1948), and a clinical diagnosis of granuloma inguinale is not complete without their observation. These intracellular bodies are now called Donovan bodies. The characteristics of the Donovan bodies have described in detail by Richens (1991) (Table IV).

2.5.1 TAXONOMY OF *CALYMMATOBACTERIUM GRANULOMATIS*

Since its recognition, the classification of *C. granulomatis* has not been resolved. This is mainly due to the absence of a sustainable culture. Earlier American workers considered the organism as belonging to the family *Enterobacteriaceae* and genus *Klebsiella* (Friedlander) because the morphological characteristics, particularly the prominence of a capsule and antigenic similarities between the two organisms (Rake, 1948). The organism had been placed in the family *Brucellaeceae* (Dienst and Brownell, 1984) in an earlier

TABLE IV: CHARACTERISTICS OF DONOVAN BODIES (RICHENS, 1991)

Morphology of Donovan bodies	Pleomorphic 1μm - 2μm x 0.5μm - 0.7μm. A capsule is often visible but the extent to which this takes up stain varies considerably from case to case and according to the technique used. Bipolar densities giving closed safety - pin appearance are often observed. No spore. Non-motile
Staining properties	Gram negative, well seen with RapiDiff, Giemsa, Leishman, Wright's or silver stains; poorly visualised with haematoxylin and eosin; not acid-fast
Host cell	Large mononuclear cells 20μm - 90 μm in diameter scattered throughout the dense plasma cell infiltrate of the lesion. Nucleus often oval, eccentric and vesicular or pyknotic. Vacuolated cytoplasm containing clusters of Donovan bodies which are occasionally, confined to the periphery of the phagosomes
Other sites for Donovan bodies	Occasional extracellular forms and organisms within polymorphonuclear neutrophils

edition of Bergys manual. The taxonomic relationship of *Calymmatobacterium* to other bacterial genera are not yet understood and the proposed name of *Calymmatobacterium granulomatis* by Aragao and Vianna (1913) has been accepted and is currently regarded as an organism of uncertain affiliation and not assigned to any genus with any established family. Only one species has been described, the characteristics of which are presented in table V.

2.5.2 ANTIGENIC CHARACTERISTICS

Although a number of immunologic tests have been reported for the diagnosis of granuloma inguinale, no specific antigenic determinants / epitopes have been recognised to elicit these immunological responses.

Using the whole bacterial cell as antigen, McIntosh (1926) observed an antibody response on the basis of agglutinins, precipitin changes in globulins and skin sensitivity in spontaneous and experimental cases. Anderson *et al* (1945) used an antigen consisting of the mucinous substance of the embryonic yolk culture and demonstrated complement fixing antibodies. The use of encapsulated organisms as antigen elicited skin reaction (Kornblith, 1944; Chen *et al*, 1949), whilst the antigen prepared from unencapsulated organisms did not (Dulaney *et al*, 1948). Thus, the capsule appears to be the only antigenic component and to date no other components have been identified.

2.5.3 CULTURE AND ISOLATION

C. granulomatis is fastidious in its growth requirements and therefore has not been cultivated *in vitro*. Ever since the discovery of Donovan bodies in stained smears, numerous attempts have been made to culture the aetiological agent from lesions. Although some workers have claimed to have cultured the organism (Cornwall and Peck, 1925; McIntosh, 1926), other investigators have doubted the culture results and suggested that experimental lesions produced from the so-called cultures were abscesses and not true granuloma inguinale.

TABLE V: CHARACTERISTICS OF *CALYMMATOBACTERIUM GRANULOMATIS* (DIENST AND BROWNELL, 1984)

- Pleomorphic 0,5µm - 1,5µm wide X 1,0µm - 2,0µm in length,
- Gram negative
- Single or bipolar condensation
- Encapsulated
- Facultatively anaerobic
- Temperature requirements: optimum - 37^0C.
- Haemolysis : no data available
- Fastidious in growth requirements, cultivated *in vivo* in yolk sac of embryonated chick eggs.
- Antigenic structure : no data available
- Pathogenicity: humans only; major role in granuloma inguinale / Donovanosis
- Source : present in ulcerative lesions

  specific geographical distribution
- DNA base composition : not available
- Type species : *Calymmatobacterium granulomatis* (Aragao and Vianna, 1913)

  Ca.lym.ma.to.bac.te'ri.um.

  Gr. n. *calymma* mantle, sheath;

  Gr. dim. neut. n. *bacterion* a small rod;

  M.L. neut. n. *Calymmatobacterium* the sheathed rodlet
- Type strain : non available

In 1943, Anderson reported the culture in the yolk sac of chick embryos of Gram negative organisms having characteristics of Donovan bodies. However, subculture of the organism on conventional solid media was unsuccessful. Various workers subsequently reported the cultivation of the presumed organism in embryonated eggs (Anderson *et al*, 1945a; Sheldon *et al*, 1945; Beveridge, 1946; Jennison *et al*, 1947; Dienst *et al*, 1948; Thomison, 1951; Dulaney *et al*, 1948; Goldberg, 1959) and in media with or without the addition of embryonic yolk; namely Locke-yolk medium (Dulaney *et al*, 1948), beef heart infusion broth, rabbit blood agar or broth and Levinthals beef heart medium (Rake and Oskay, 1948) and fresh egg yolk agar medium (Dienst *et al*, 1948). The fresh yolk medium of Dienst *et al* (1948) was modified by replacing the egg yolk with lactalbumin hydrolysate and Anderson's originally passaged strain was said to have been cultured in 1959 (Goldberg, 1959). Sodium azide and brilliant green were added to this medium to make it selective (Goldberg, 1962). However, *C. granulomatis* growing in these artificial media did not retain the normal capsule and did not grow luxuriantly. It is at present unclear how many of these claims are true.

2.5.3.1 Identification

Identification of microorganisms is usually based on colonial morphology, Gram stain reaction, biochemical tests and susceptibility to certain drugs and dyes. Since *C. granulomatis* is the organism implicated in the aetiology of granuloma inguinale it is necessary to distinguish it from other organisms which commonly occur as contaminants or bacterial superinfection in ulcerative lesions.

The identification of *C. granulomatis* has been based on the recognition of the typical morphology of the organism in stained smears. The two characteristic features of the organism are extreme pleomorphism and intracellular parasitism in mononuclear cells. When stained by Gram's method the organism appears as a rod without a capsule and is difficult to distinguish from other bacilli. With the Wrights' stain, the capsule is pink and homogenous and the bacterial body shows bipolar condensation of chromatin material. Anderson *et al* (1945a) reported on the appearance of the organism from harvested egg yolk, the characteristic feature of pleomorphism was prominent, both encapsulated and

unencapsulated forms were present and the capsule appeared to be thicker and wider as compared to bacteria seen in direct specimens.

From the earlier reports, the organism was found not to grow aerobically and anaerobically on solid media and its growth in egg yolk thioglycollate medium suggested that a reduced redox potential was required. In this microaerophillic environment there was evidence of the organism increasing in length (Goldberg, 1959, 1962). Growth occurred between 20°C and 40°C, with the optimal temperature being 32°C. The organism has been shown to survive at room temperature in embryonic yolk culture (Anderson *et al*, 1945a) and in Locke-yolk culture for ten weeks (Dulaney *et al*, 1948), whilst it was suppressed at below 20°C and ceased to grow at 5°C. A temperature of 40°C inhibited multiplication and a temperature of 56°C for 30 minutes was sufficient to kill all viable forms. The optimal pH for growth was 7,3, whilst it was shown to survive at between pH 5,0 to 7,8.

The action of various chemical compounds against *C. granulomatis* in the embryonic yolk culture has also been reported (Beveridge, 1946). The organism was inhibited by trivalent antimony (tartar emetic, anthiomaline, or fuadin) whilst ten times as much pentavalent antimony (neostam or sodium stibogluconate) was required to produce the same effect. Rake and Dunham (1947) tested an extensive list of substances by allowing the culture to contact the substance in solution for two hours before inoculating it into embryonated eggs. The concentration at which 50% of the embryos were killed was taken as the end point. Mercuric chloride was found to be the most effective disinfectant against *C. granulomatis*, whilst fuadin and tartar emetic were less effective. Streptomycin was found to be more effective than penicillin G and twice as more potent than tartar emetic.

Dienst *et al* (1948) tested the action of various antibiotics against *C. granulomatis* in fresh yolk agar medium. The bacteriostatic concentration of streptomycin was 0,075 mg/L and 0,15 mg/L for four and two day old cultures respectively. The bactericidal concentration for two day old culture for streptomycin was 0,2 mg/L; for penicillin 0,5 mg/L; for aureomycin 5,0 mg/L; for chloromphenicol 10,0 mg/L and for bacitracin 600,0 mg/L. A standard acqueous solution of podophyllin did not have an inhibitory effect on the organism in culture.

Carbohydrate fermentation tests have been performed by adding one percent of various sugars to the yolk agar medium and incubating the inoculated tubes with their corresponding uninoculated control tubes at 37° C for 48 hours. Fermentation of dextrose, mannitol and maltose occurred, but not for lactose or sucrose. The tests for indole and hydrogen sulphide production were recorded as negative. These results have not been reproducible (Dienst *et al*, 1949).

2.6 GRANULOMA INGUINALE AND HUMAN IMMUNODEFICIENCY VIRUS INFECTION

Sexually transmitted diseases in general and genital ulcerative diseases in particular have been shown to be important risk factors for the transmission of the human immunodeficiency virus (HIV) (Greenblatt *et al*, 1987; Wasserheit, 1992; Clottey and Dallabetta, 1993). The proposed mechanism to explain how genital ulcerative disease could facilitate the transmission of HIV is by the increased shedding of the virus through the ulcer (Kreiss *et al*, 1989; Plummer *et al*, 1991) or the presence of an ulcer results in the disruption of the epithelial barrier with bleeding which increases the number of susceptible cells at the point of entry, thereby increasing the susceptibility to HIV infection.
CD4 - T lymphocytes have been identified in syphilitic (Engelkens *et al*, 1993), herpes genitalis (Cunningham *et al*, 1985) and granuloma inguinale (Sehgal *et al*, 1991a) lesions. Using monoclonal antibodies Sehgal *et al* (1991a) estimated total T, B, CD4 and CD8 - lymphocyte populations in tissue sections of lesions from 22 granuloma inguinale patients, comprising 17 ulcerogranulomatous, four hypertrophic and one sclerotic variant. The CD4:CD8 ratio in the ulcerogranulomatous variants was 1.41 which was significantly higher than the hypertrophic variants (1.28). The presence of these cells having the CD4 antigen receptor for HIV would therefore promote the transmission and acquisition of HIV at the site of the lesion.

O' Farrell (1991) reported that among men with granuloma inguinale, ulceration of six weeks or longer duration was associated with HIV 1 seropositivity, with the level of statistical significance being greatest in men with lesions of more than 12 weeks duration. The lesions of granuloma inguinale are painless and therefore do not preclude sexual

intercourse despite bleeding, thus the longer the period of ulceration the greater the likelihood of intercourse occurring with exposure and transmission of HIV (O'Farrell *et al*, 1992).

In patients with acquired immunodeficiency syndrome (AIDS) and granuloma inguinale, there is an atypical evolution and enlargement of lesions, despite the appropriate therapy used (Jardim *et al*, 1990). Hoosen *et al* (1996) reported in their series, 16% (18/110) of patients with granuloma inguinale had antibodies to HIV. However, in this study the clinical presentation, course and response to therapy was the same as for seronegative individuals. Since the CD4 counts were not performed, the extent to which these patients were immunocompromised could not be established. These workers attributed the unaltered course to the fact that seropositive patients were not necessarily immunocompromised as none of their 18 patients had any clinical features of AIDS.

CHAPTER 3.0
CULTURE OF *CALYMMATOBACTERIUM GRANULOMATIS*

3.1 INTRODUCTION

Since Donovan first described the encapsulated micro-organism (Donovan, 1904; 1905) it is now generally recognised as being constantly present in lesions of granuloma inguinale. However, there has been wide diversity of opinion as to the part played by this organism in the causation of the disease. Some early investigators were of the opinion that the Donovan organisms were not themselves responsible for the disease, but were found in lesions in association with some other organism, and probably acted in symbiosis. However, there were others who considered the Donovan organism to be the true aetiological agent (Anderson *et al*, 1945a).

Currently the laboratory diagnosis of granuloma inguinale relies on the observation of Donovan bodies in tissue smears or biopsy specimens utilising the Giemsa and Wright's stains (Anderson *et al*, 1945a; Cannefax, 1948). To increase the sensitivity, specificity and the rapidity of diagnosis Dieterlies, Warthin-Starry (Greenblatt and Barfield, 1952) and the rapid Giemsa (O'Farrell *et al*, 1990) stains have been used. Conventional culture methods for the laboratory identification of the causative organism are not available and hence the organism remains poorly characterised.

Many attempts have been made to cultivate the Donovan organism *in vitro* since its discovery. In 1911, Flu (cited by DeMonbreun and Goodpasture, 1931) isolated a Gram negative, non motile, encapsulated bacillus from a patient with granuloma inguinale. In 1913, Aragao and Vianna (1913) reported isolating the organism in pure cultures from several patients. They considered the morphological characteristics to be the same as that of organisms found in lesions and named the aetiological agent as *Calymmatobacterium granulomatis*. Walker in 1916, cultured an encapsulated bacillus which he classified as belonging to the *Bacillus mucosus capsulatus* group of Friedlander. The findings of this study were corroborated by Randall *et al*, (1918) who

cultivated a similar organism from three of their twelve cases. Numerous investigators have reported the isolation of Gram negative encapsulated organisms representing Donovan bodies (McIntosh, 1926; Goldzieher and Peck, 1926; Campbell, 1927). Surprisingly, all isolates had been reported to have been cultured on Sabourauds media, which is primarily used for the cultivation of yeasts. John and Gage (1926) (cited by DeMonbreun and Goodpasture, 1931), after conducting extensive culture studies were unconvinced that the Donovan organisms had ever been cultured. Later Gage (1929) reported that the organism with morphological characteristics of Donovan organisms was not identical to *Bacillus mucosus capsulatus* and Castellani and Mendelson (1929) assigned them to the group *Bacillus lactis aerogenes, genus encapsulatus.* In 1931, DeMonbreun and Goodpasture (1931a) reported to have cultured the organism in inactivated sera of patients, but always in association with a streptococcus and the presumed organism was found to grow best in ascitic fluid to which egg yolk had been added. These workers failed to cultivate the Donovan organism on a number of artificial cell free media. Until 1939 the organism was still thought to be a protozoan (Greenblatt *et al*, 1939). There were doubts whether the aetiological agent of granuloma inguinale had truly been cultured. No real progress has been made with regard to culture and the sustainment of the aetiological agent in any type culture collection.

The isolation of the organism in the yolk sac of fertile chick eggs was reported by Kathleen Anderson in 1943 (Anderson, 1943). Pure growth of a Gram negative bacillus with close morphological similarities to the bacteria seen in smears of granuloma inguinale lesions was obtained. This work established the bacterial nature of the Donovan bodies. However, these isolates failed to grow on any conventional liquid or solid cell free media. Thereafter, between 1943 and 1951 a few reports appeared on the attempts on primary isolation of the organism in yolk sac of fertile chick eggs and the subsequent adaptation of yolk sac cultures to artificial media (Anderson *et al*, 1945a, Sheldon *et al*, 1945, Beveridge, 1946; Jennison *et al*, 1947; Dienst *et al*, 1948; Thomison, 1951; Dulaney *et al*, 1948; Goldberg, 1959; 1962). Early reports on the cultural characteristics of *C. granulomatis* are detailed in table VI. Despite the reports of these early successes, there have been no subsequent reports on the growth and characterisation of this organism.

In general, culture media are formulated for the cultivation of micro-organisms, which may be for the purposes of isolation, identification, susceptibility testing and characterisation of micro-organisms. As living cells, micro-organisms require nutritional sources of nitrogen, carbon, salts and other nutrients. These are usually derived from external sources, however some micro-organisms are incapable of surviving outside living cells, despite the availability of these specific requirements and are strict parasites of other living cells.

Monocytes and macrophages are bone marrow derived leukocytes that infiltrate almost every tissue of the body, where they acquire different properties that allow them to perform a variety of functions. These cells together with neutrophils provide the phagocytic defense mechanism, protecting the host against microbial invasion. When the macrophage is activated, intracellular and extracellular pathogens may be inhibited or destroyed by the generation of toxic oxygen metabolites (Loms Ziegler-Heitbrock, 1989). However, certain pathogens have chosen macrophages as their preferred habitat and parasitise these cells, replicate within them and their continued presence, results in persistent activation of monocytes and a chronic inflammatory response with ensuing tissue damage and granulomatous disease (Kauffman and Flesch, 1992).

Many facultative intracellular pathogens such as *Listeria monocytogenes, Shigella flexneri, Salmonella species, Mycobacterium tuberculosis* and *Legionella pneumophilia* grow well on artificial cell free media as well as within eukaryotic cell lines. Some micro-organisms such as *Chlamydia* and *Erlichia* being obligate intracellular parasites require living host cells to support their growth. Thus, the introduction of tissue culture isolation procedures for fastidious micro-organisms are relevant for the culture and growth enhancement of such micro-organisms.

TABLE VI: EARLY REPORTS ON CULTURE MEDIA, INCUBATION CONDITIONS AND CULTURAL CHARACTERISTICS OF *CALYMMATOBACTERIUM GRANULOMATIS*

Reference	PC/SC	Specimen	Medium	L, SS, S	Temp	Incubation in Days	Morphological Characteristics
Anderson, 1943	PC	GT	a. 8d yolk sac chick embryo b. 5d chick embryo yolk with chick heart	L L	 37^{0}C	8d 6d	Gram negative, pleomorphic, encapsulated and non encapsulated forms. good growth, same as above
Anderson *et al*, 1945	PC	GT	a. 5 - 6d chick embryo	L	37^{0}C	8d	Gram negative, pleomorphic, initially non encapsulated, progressing to good growth of both encapsulated and non encapsulated forms; encapsulated - large capsule, safety pin appearance non - encapsulated - small delicate bacilli
Beveridge, 1945	PC	GT	a. yolk sac b. yolk sac with penicillin	L	37^{0}C	6 - 10d	Gram negative Bipolar staining reaction with Leishman stain
Sheldon *et al*, 1945	PC	a. GT b. BM	6d chick embryo	L	37^{0}C	2 - 11d	Gram negative Short, encapsulated, safety pin appearance with Wrights stain
Jennison *et al*, 1947	PC	GT	5d chick embryo	L	37^{0}C	11d	Highly pleomorphic, gram negative rod. Encapsulated, intense bipolar and safety pin forms with Wright stain. Non motile, viable at room temperature in harvested yolk for four weeks, but not ten weeks
Dienst *et al*, 1948	SC	N/A	fertile / unfertile a) unheated yolk b) heated (60^0) yolk c) fresh yolk with 0,12% agar	 L S SS	 37^{0}C 37^{0}C 37^{0}C	 4d	All media showed characteristics of Donovan bodies as seen in direct tissue smears
Dulaney *et al*, 1948	SC	N/A	a) Dulaneys medium : 50% yolk from 5 day fertile eggs with Lockes solution b) as in (a) with $^1/_{10}$ normal human serum	S S	37^{0}C 37^{0}C	2d 2d	Curved bacillary forms with bi, central or multiple granules. Safety pin appearance common, capsule absent, no flagella as above with chain formation and a variety of bizarre pleomorphic forms

Rake and Oskay, 1948	SC	N/A	a) Levinthals beef heart infusion agar	S	37°C	2d	Translucent, shiny colonies grey to brown, 1,5mm in diameter; mucoid in earlier passages
			b) Rabbits blood agar	S	37°C	11d	Translucent, shiny colonies grey to brown, 1,5mm in diameter; mucoid in earlier passages
			c) Rabbits blood broth	L	37°C	2d	Growth scanty, clearing in tubes indicating lysis of organisms
			d) Beef heart infusion broth	L	37°C	2d	Growth scanty, clearing in tubes indicating lysis of organisms
Dunham and Rake, 1948	SC	N/A	a) Beef heart infusion agar with 10% yolk from 6d fertile eggs	S	35°C	2d	Colonies : irregular, shiny, translucent, grey, mucoid Gram negative
			b) Beef heart infusion broth with 3% agar and modified Levinthals broth	S			Deep blue - bipolar staining Characteristic safety pin appearance curved bacilli, pleomorphic
Thomison, 1951	PC	GT	Intra cerebral inoculation into 1d old chick	N/A	37°C	14d	Small Gram negative bacilli bipolar staining
Goldberg *et al*, 1953	SC	N/A	Dulaney medium : 5●% yolk from 5d fertile eggs in Lockes solution	S	37°C	5d	Variation in morphology of the different strains. non motile, non capsulated, thin, slender gram negative pleomorphic.
Goldberg, 1959	SC	N/A	Lactalbumin hydrolysate with NaCI, Dipotassium phosphate sodium thioglycollate L-cystine	L	NR	NR	Media for maintenance of organism
Goldberg, 1962	PC	F	Lactalbumin hydrosylate with sodium azide and brilliant green	L	37°C	4d	Gram negative, non spore forming Bipolar staining with Wrights stain. Pleomorphic with variation in morphology with each transfer

et al	and others	GT	granulation tissue	L	liquid medium	NR	not reported
PC	Primary cultivation	BM	bone marrow	SS	semi solid medium	NA	not applicable
SC	Secondary cultivation	F	faeces	S	solid medium		
				d	day/s		

The ability to grow normal human monocytes by addition of exogenous growth factors and continued propagation for several weeks is possible. This has been of great biomedical importance since it has allowed the growth of microbial pathogens within monocytes and macrophages which has provided an opportunity to study host - parasite interactions, phagocytic and microbicidal activity of macrophages and the role of macrophages in the pathogenesis of disease (Furness, 1958; Morello and Baker, 1965; Lissner *et al*, 1983). The choice of the cell source is critical for the establishment of growth by a particular pathogen. This is dependent on the physiological state of the cell line and the distribution of receptors that are necessary for bacterial adherence, invasion and establishment of growth. In this study monocytes were used to allow growth of the organism since these cells are the target cells in the host tissue.

The aminoglycoside, gentamicin is frequently used for the measurement of eukaryotic cell penetration by bacteria (Elsinghorst, 1979). Aminoglycosides inhibit protein synthesis by combining with bacterial proteins of the 30S ribosomal subunit. This binding is irreversible and blocks the initiation complexes of peptide formation. The mRNA is misread on the recognition region of the ribosome and as a result an incorrect amino acid is inserted into the peptide resulting in the formation of non - functional proteins resulting in a rapid bactericidal effect. Because of the limited penetration into eukaryotic cells (Vaudaux and Waldvogel, 1979), organisms that penetrate the eukaryotic membrane are protected from the bactericidal effects of the aminoglycoside, whereas susceptible extracellular organisms are rapidly killed by the antibiotic. The mechanism of action of aminoglycosides allows for the *in vitro* measurement of events such as invasion, intracellular survival and replication, as well as intracellular spread.

For the treatment of severe granuloma inguinale, aminoglycosides have been used (Greenblatt *et al*, 1947; Maddocks *et al*, 1976; Hoosen *et al*, 1996). Streptomycin has been used previously with good results (Greenblatt *et al*, 1947). More recently gentamicin (Maddocks *et al*, 1976) and amikacin (Hoosen *et al*, 1996) have been prescribed also with good effect.

Since the Donovan bodies are found within mononuclear cells, the use of an

aminoglycoside, such as amikacin was considered for decontamination of tissue specimens collected for propagating *C. granulomatis*. It was assumed that amikacin would "kill" all the susceptible extracellular organisms, especially the numerous contaminants present in genital ulcer specimens, including extracellular Donovan bodies and allow the survival of intracellular organisms. With the removal of suspension medium which consists of amikacin and by placing in an environment of mononuclear cells without amikacin, the organisms from the specimen would invade macrophages and establish growth.

The aims of this study were to

- cultivate *C. granulomatis*, the aetiological agent of granuloma inguinale using a monocyte co-culture technique.

- determine the morphological characteristics of *C. granulomatis* in culture

3.2 MATERIALS AND METHODS

3.2.1 DEVELOPMENT OF CULTURE SYSTEM

Over a three year period all female patients with ulcerative genital lesions, presenting to the antenatal and gynaecology outpatient clinics of King Edward VIII Hospital, Durban were recruited for the study. Smears and / or tissue biopsy specimens were obtained from ulcerative lesions and investigated for the causative agents of chancroid, herpes genitalis, lymphogranuloma venereum, syphilis and granuloma inguinale using conventional laboratory tests. All Donovan body positive biopsy specimens were processed for co-culture.

3.2.2 CULTIVATION METHODS

3.2.2.1 Isolation of peripheral blood mononuclear cells

The collection, separation and isolation for peripheral blood mononuclear cells (PBMNC) was based on the methods described by Boyum, (1968); Johnson *et al* (1977) and Adams (1979) with modifications. All procedures were carried out under aseptic conditions. Thirty millilitres of peripheral whole blood from a single normal healthy volunteer was withdrawn into a heparinized tube. The tubes were gently rotated to avoid clotting. The blood was diluted with an equal volume of prewarmed Hanks' balanced salts solution (HBSS) (Whittaker M.A. Bioproducts, Maryland, USA). This mixture was carefully layered onto prewarmed Histopaque 1077 (Sigma Chemicals, St Louis, MO, USA) in a ratio of 5:4 in a sterile 50 ml test tube (Bibby Sterilin Ltd, UK). The tubes were transferred to a Beckman centrifuge and spun at room temperature at 2000 rpm for 30 minutes. Using a sterile Pasteur pipette, the mononuclear cells were carefully aspirated from the interface between the HBSS and the Histopaque, and transferred to a sterile centrifuge tube. The mononuclear cells were washed two times in HBSS and centrifuged each time for 15 minutes at 1200rpm at room temperature to remove any residual Histopaque. The cell pellets were resuspended in one ml RPMI 1640 (Whittaker M.A. Bioproducts, Maryland, USA) supplemented with 2mM L - glutamine

and 10% unheated autologous serum.

3.2.2.1.1 *Cell counts*

Cell suspensions were diluted with RPMI 1640 (1 in 10). Using sterile pipettes the suspensions were carefully added to both the chamber sides of the haemocytometer. The chamber coverslips were added and the appropriate squares counted at x100 magnification. After counting the original suspension was diluted and adjusted to 5 x 10^6 cells/ml in culture medium.

3.2.2.2 Establishment of monocyte monolayers for cultivation

One millilitre volume of the PBMNC (5 x 10^6 cells/ml) were transferred to tract vials containing glass coverslips (Bibby Sterilin Ltd, UK) to which the monocytes were allowed to adhere for one hour at 37°C in 5% CO_2 in air. The non adherent cells from each vial were removed by extensive washing with prewarmed supplemented RPMI 1640 medium. Before inoculation with the tissue specimen, the viability and integrity of the monocytes were measured by the trypan blue exclusion test (Patterson, 1979) and by direct observation under an inverted microscope (Patterson, 1979). Greater than 95% of adhering cells on the coverslips were monocytes as confirmed by the esterase test (Yam *et al*, 1971).

3.2.2.2.1 *Trypan blue stain*

This test is based on the ability of an intact cell membrane to exclude certain dyes such as safranin, eosin, Congo red, alcian blue nigrosin and trypan blue (Patterson, 1979). Cell suspensions were prepared by diluting 9µl of cell suspension and 1µl of trypan blue dye. After 5 minutes, a drop of suspension was placed on a glass slide, covered with a coverslip and examined by light microscope at x400 magnification. Colourless cells were considered to be viable, whilst the blue cells were regarded as non - viable. The cells were harvested if the dye exclusion test showed a viability count of greater than 95%.

3.2.2.2.2 *α-Naphthyl acetate esterase stain*

The cytologic demonstration of α-naphthyl acetate esterase activity in monocytes was determined by the α-naphthyl acetate esterase reagent kit (Sigma Diagnostics, St, Louis, MO, USA). This method distinguishes granulocytes from monocytes when cells are incubated with α-naphthyl acetate in the presence of a diazonium salt. Enzymic hydrolysis of ester linkages liberate free naphthol compounds which couple with the diazonium salt, forming highly coloured deposits at the sites of enzymic activity.

Smears of prepared monocytes/macrophages were made onto slides, allowed to dry for one hour, fixed in citrate-acetone-methanol fixative for one minute at room temperature and washed thoroughly in deionised water. The slides were air dried for 20 minutes. One capsule of Fast Blue RR salt was added to 50 mls of prewarmed Trizmal 7,6 dilute buffer solution with constant stirring. When the salt had completely dissolved, two ml α-naphthyl acetate solution was added. The solution turned yellow and slightly turbid. The solution was stirred for a further 15 - 20 seconds and poured into a coplin jar. The slides were placed in this staining solution and incubated at 37°C for 30 minutes. The slides were removed from the stain and washed for 3 minutes in deionised water and counterstained with Mayer's haematoxylin for 5 - 10 minutes and washed in tap water. The slides were air dried and examined microscopically.

Cells were considered to be monocytes on the basis of the presence of the α-naphthyl acetate esterase enzyme which was easily detected and appeared as black granulation within the cytoplasm. In the co-culture system cells were initially used in the monocyte phase with an esterase value of >95%.

3.2.3 PREPARATION OF INOCULUM FROM BIOPSY SPECIMENS

Twenty two biopsy specimens were suspended in prewarmed supplemented RPMI 1640. Amikacin (Bristol Laboratories, UK) was added at a concentration of 10 mg/L and incubated at room temperature for two hours and thereafter rinsed in phosphate buffered saline pH 7,2. Using a Dounce tissue grinder, the specimens were homogenised and

suspended in prewarmed supplemented RPMI 1640. Smears were prepared on glass slides, air dried and examined by the RapiDiff stain (O'Farrell *et al*, 1990) to estimate the number of organisms before inoculation onto monolayers.

3.2.3.1 RapiDiff stain

The RapiDiff stain (Clinical Science Diagnostics Ltd, Booysens 2016, South Africa) was used to determine the presence of both intracellular and extracellular organisms. The prepared glass slides of homogenised tissue smears were air dried and placed in RapiDiff fixative for 15 seconds, then placed in solution RapiDiff 1 (Eosin Y) for 15 seconds and thereafter placed in solution RapiDiff 2 (Thiazine Dye mixture) for 15 seconds and finally rinsed in distilled water. The stained slides were air dried, mounted with DPX mountant (BDH Laboratories Supplies, UK) and examined by light microscopy under oil immersion (x1000 magnification). The Donovan bodies stained as pink - purple bacillary bodies with characteristic intense staining blue - purple bipolar condensation. The capsules were usually unstained appearing as a halo or occasionally having a light pinkish irregular outline around a deeply stained bacillary body.

3.2.4 INOCULATION AND INFECTION OF MONOCYTE MONOLAYERS

The uninfected monolayers were incubated for 20 hours afterwhich the RPMI was aspirated with a pipette. The track vials were inoculated in triplicate with 500µl of the infected biopsy specimen homogenate. After incubation for one hour at 37°C in 5% CO_2 in air, one millilitre of fresh supplemented RPMI 1640 without antibiotics was added, followed by incubation for another 48 hours. Subcultures were made onto fresh monocyte monolayers which had been incubated for 20 hours. The coverslips and suspensions were examined by RapiDiff, Gram, indirect immunofluorescence, electron microscopy and DNA analysis respectively.

3.2.5 SUBCULTURES ON CELL FREE MEDIA

In order to check for contaminating flora (table VII) and to determine whether any cell free media would support the growth of *C. granulomatis*, a variety of culture media (table VIII) were inoculated with homogenised biopsy specimens and monocyte co-culures and incubated at appropriate temperatures and atmospheric requirements for a period of seven days.

3.2.6 MODIFICATION OF THE CO-CULTURE METHOD

A series of experiments were carried out in an attempt to set up the appropriate conditions for the growth of *C. granulomatis* by co-culture. Modifications were made both in the preparation of the inoculum and to the cell line.

3.2.6.1 Modified method for collection of mononuclear cells

As the availability of blood from laboratory volunteers was restricted, monocytes from pooled HIV negative blood donors attending the Natal Blood Transfusion Services were used. Buffy coat fractions were mixed with an equal volume of prewarmed unsupplemented RPMI 1640 and transported to the laboratory and immediately subjected to a density gradient as described in section 3.2.2.1.

3.2.6.2 Modified method for preparation of tissue culture coverslips

Round glass coverslips (1 x 13mm, Chance Propper,UK) were autoclaved for 15 minutes at 121°C. Prior to use coverslips were carefully transferred to wells of a sterile 24 well tissue culture plate (Falcon - 3047; Becton Dickinson, New Jersey) and exposed to ultraviolet light (254nm) for 30 minutes.

3.2.6.3 Modified method using foetal calf sera

The autologous serum used in the initial experiments was replaced with foetal calf serum

TABLE VII: CULTURE MEDIA AND INCUBATION CONDITIONS USED TO CHECK MONOCYTE CO-CULTURES FOR THE PRESENCE OF CONTAMINATING FLORA.

MEDIUM	INCUBATION TEMPERATURE
Blood agar	25°C, 33°C, 37°C
MacConkey agar	37°C
Chocolate agar	37°C
Sabourauds agar	25°C, 37°C
Brucella agar	25°C, 37°C
10% Blood agar	25°C, 33°C, 37°C
Thioglycollate broth	25°C, 37°C
Mueller Hinton agar with 1% isovitalex, 3µg/ml vancomycin and 5% chocolatised horse blood	33°C, 37°C
Wilkens Chalgren agar	37°C

TABLE VIII: CELL FREE CULTURE MEDIA USED FOR THE ATTEMPTED RECOVERY OF *CALYMMATOBACTERIUM GRANULOMATIS* HOMOGENISED TISSUE BIOPSY SPECIMENS AND MONOCYE CO-CULTURES

MEDIUM	INCUBATION TEMPERATURE	REFERENCE
Dulaneys slants	25°C, 37°C	Dulaney *et al,* 1948
*modified: 50% egg yolk in thioglycollate medium and thioglycollate overlay	25°C, 37°C	This study
*modified: 50% egg yolk in Lockes solution and thioglycollate overlay	25°C, 37°C	This study
*modified: 50% egg yolk in thioglycollate medium and Lockes solution overlay	25°C, 37°C	This study
*modified: 50 % egg yolk in Lockes solution and 1/10 normal human sera and Lockes solution overlay	25°C, 37°C	This study
Lactalbumin hydrolysate (LH)	25°C, 37°C	Goldberg, 1959
*with sodium azide and brilliant green	25°C, 37°C	Goldberg, 1962
*with activated charcoal	25°C, 37°C	Goldberg, 1962
*LH replaced with phytone peptone	25°C, 37°C	Goldberg, 1962
*LH replaced with yolk from 5 to 7 day embryo	25°C, 37°C	Goldberg, 1962
Embryonic yolk	25°C, 33°C, 37°C	Dienst *et al*, 1948
*with macerated chick heart	25°C, 33°C, 37°C	Anderson *et al*, 1945
Cystine agar slants	25°C, 33°C, 37°C	Anderson, 1943

* indicates modifications to culture media

(Delta Bioproducts, South Africa), because of the inaccessibility of autologous serum when pooled cells from blood donors were used. The foetal calf serum had been heat inactivated at 56°C for 30 minutes.

3.2.6.4 Modified method for decontamination of biopsy specimens

To establish which antibiotics could be used adequately for specimen decontamination, all contaminating flora grown on conventional media were tested for their susceptibility to a variety of antimicrobial agents using the Kirby Bauer disc diffusion susceptibility method. The antimicrobial agents tested were penicillin G (6 units), ampicillin (25μg), amoxycillin/clavulanic acid (30μg), oxacillin (1μg), cephalothin (30μg), cefuroxime (30μg), cefoxitin (30μg), cefotaxime (30μg), ceftazidime (30μg), kanamycin (10μg), gentamicin (10μg), amikacin (30μg), chloramphenicol (10μg), tetracycline (10μg), erythromycin (15μg), clindamycin (10μg), vancomycin (30μg), ciprofloxacin (5μg), imipenem (10μg) and metronidazole (10μg). Subsequent biopsy specimens were treated with a combination of vancomycin (5mg/L) (Eli Lilly, USA), metronidazole (10mg/L) (Rhone-poulenc, UK) and amikacin (10mg/L) (Bristol Laboratories, UK) for two hours and this combination was found to be most adequate for specimen decontamination.

These modified techniques, viz. the use of mononuclear cells from voluntary blood donors, 24 well tissue culture plates containing glass coverslips, foetal calf serum and the improved method of decontaminating tissue specimens were applied and used to culture subsequent biopsy specimens from female patients as well as tissue scrapings obtained from male patients presenting with genital ulcer disease to the Sexually Transmitted Diseases (STD) Clinic, City Health, Durban.

3.2.7 APPLICATION OF MODIFIED METHOD

3.2.7.1 Preparation of biopsy specimens for inoculation

Nineteen additional biopsy specimens were obtained from female patients presenting with genital ulcer disease. These were treated with amikacin (10mg/L), vancomycin

(5mg/L) and metronidazole (10mg/L) for two hours and prepared for culture as described in sections 3.2.3 and 3.2.4.

3.2.7.2 Preparation of tissue scrapings for inoculation

In a study to determine the aetiology of genital ulcer disease amongst adult males attending the Durban STD clinic, two hundred consecutive patients with genital ulcerations were investigated. Tissue scrapings were obtained with a 100µl plastic bacteriological loop (Bibby Sterilin, Ltd, UK) and suspended in 500µl of PBS pH 7,2. One hundred microlitres were transferred to 500µl of decontamination medium containing RPMI 1640 supplemented with 10% foetal calf sera (Delta Bioproducts, SA) and amikacin (10mg/L), vancomycin (5mg/L) and metronidazole (10mg/L). After two hours the tubes were centrifuged at low speed, excess supernatant discarded and resuspended in one millilitre of RPMI 1640 supplemented with 10% foetal calf serum without antibiotics before inoculating onto mononuclear cells.

3.2.8 CONFIRMATION OF CULTURES

Infected monocyte co-cultures were subjected to the following. Each of the triplicate vials was used for RapiDiff, Gram, indirect immunofluorescence, electron microscopy and DNA analysis.

3.2.8.1 Growth observations

For macroscopic observations, the medium was checked daily for colour changes indicating change in pH. For microscopic observations, the medium was removed from the monolayers and the coverslips were fixed in methanol and stained with RapiDiff and Gram's stains. The size of the bacteria were estimated using the Kontron 100 image analysis programme (Kontron, Germany) linked to a Nikon Biophot photomicroscope.

3.2.8.2 Indirect immunofluorescence

Smears of biopsy specimens and cells from all positive monocyte co-cultures were scraped off the glass coverslips, centrifuged and the deposits placed on the clear well of a glass slide and allowed to air dry. All such smears were fixed with methanol and air dried. For positive controls, smears were obtained from ulcerative lesions showing characteristic Donovan bodies. For negative controls, laboratory strains of *Klebsiella pneumoniae, K. oxytoca, K. aerogenes, Enterobacter cloacae, Citrobacter freundii* and *Escherichia coli* were used. All slides were treated with sera from two patients with granuloma inguinale and serum from a volunteer with no present or past history of genital ulcer disease. Sera were tested at dilutions of 1 in 80 and 1 in 160. The slides were incubated for 30 minutes at 37°C in a humidified chamber and then washed with phosphate buffered saline (PBS) pH 7,4 and reincubated with fluorescein isothiocyanate labelled mouse antihuman IgG (1:100) (Wellcome Diagnostics, UK) for a further 30 minutes. The cells were rinsed in two washes of PBS for 10 minutes each and air dried and mounted in PBS-glycerol. These were visualised using an Olympus fluorescent microscope at a magnification of x1000. A result was considered positive when characteristically brightly staining green Donovan bodies were visualised as compared to the Donovan bodies seen in direct smears of lesions. Negative results were the absence of fluorescence in the smears of bacterial cultures.

3.2.8.3 Transmission electron microscopy

Transmission electron microscopy was used to confirm morphological characteristics of the organisms in the monocyte co-culture system. The method and results of transmission electron microscopy findings are presented in chapter 4.0

3.2.8.4 Amplification and sequencing of 16S rDNA

Chromosomal DNA was extracted from the positive monocyte co-cultures and the bacterial 16S ribosomal DNA was amplified using the polymerase chain reaction with universal bacterial primers. The amplified product was sequenced. The method and

results are presented in chapter 5.0.

3.2.9 GROWTH KINETICS

The numbers of organisms in the monocyte co-cultures were estimated by counting the bacteria in fixed stained coverslips of monocyte co-culture preparations. The total number of *C. granulomatis* in monocyte co-cultures were quantitated by taking a mean of the number of extracellular and intracellular organisms counted at a magnification of x1000 over ten different fields per coverslip. During this period the moncyte co-cultures were not replaced with fresh medium. This experiment was performed on three cultures and the coversilps were stained at time intervals of 24 hours. To propagate the aetiological agent, passaging was carried out every 48 hours.

3.3 RESULTS

3.3.1 EFFECTS OF GROWTH ON CULTURE MEDIUM

The medium used for the cultivation of Donovan bodies was RPMI with unheated autologous or heat inactivated foetal calf sera. Throughout the growth phase the pH of the medium remained at pH 7,0 increasing slightly after 48 hours. Up to 48 hours there was no obvious turbidity of the medium indicating growth. However, at 72 hours there was slight turbidity. The growth of the mononuclear cells and the organisms was best at pH 7,0. Growth was evident at pH 6.0, however, at pH greater than 7,0 (when medium changed colour to intense pink) the growth of the bacteria was diminished.

3.3.2 RECOVERY OF ORGANISMS FROM CLINICAL SPECIMENS

Initially a total of 22 tissue biopsy specimens were cultured in the monocyte co-culture system. Pure cultures were obtained from three biopsied specimens. The remaining nineteen cultures were overgrown with a variety of microorganisms, despite amikacin treatment. In 12 of the 19 cultures bacteria, morphologically resembling *C. granulomatis* were visualised amongst the mixed cultures.

From the nineteen additional biopsy specimens cultured, six yielded pure growth after use of the modified method. Fourteen of the two hundred scrapings from men presenting with genital ulcers to the STD Clinic yielded growth in monocyte co-cultures after 48 hours incubation. None of these organisms grew on any of the cell free culture media used to check for the presence of contaminating flora or for the recovery of *C. granulomatis*.

3.3.3 CONFIRMATION OF CULTURES BY IMMUNOFLUORESCENCE

All monocyte co-cultures ie. the nine from biopsy specimens and 14 from tissue scrapings tested positive with the patients sera and negative with the serum from the volunteer at both dilutions (1:80 and 1:160) by the indirect immunofluorescence test.

Fluorescent bacilli were seen in association with the monocytes (Figure 8) and individually (Figure 9). The morphology of the bacilli was coccobacillary (Figure 8) or slender and tapered (Figure 9).

Direct smears from lesions with Donovan bodies were positive (Figure 10) with patients sera and negative with serum from the volunteer, whilst the smears of the control bacterial cultures were all negative with both sets of sera (Figures 11A and 11B).

3.3.4 MORPHOLOGY OF THE ORGANISM

3.3.4.1 Morphology of the organism in direct specimens

From direct smears the Donovan bodies were pleomorphic, found predominantly intracellularly within macrophages (Figure 12). The infected macrophages were enlarged with the nuclei displaced to the periphery of the cell cytoplasm. The bacteria were present within the multiple vacuoles of the cytoplasm (Figure 13). The morphology of the organism varied being either coccobacillary and bacillary, displaying pleomorphism. Some bacilli appeared to be slender with tapering ends, whilst others were short and fat with an intense staining reaction. Both encapsulated and non-encapsulated forms were present. The encapsulated forms were recognised by the presence of dense, pinkish, hazy material around the organism when stained by the RapiDiff stain.

3.3.4.2 Morphology of the organism in culture

The coverslips from each vial were air dried and examined after staining by Gram and RapiDiff stains. After 48 hours incubation with monocytes, the bacteria were visualised both intra (Figure 14) and extracellularly (Figure 15). The intracellular bacteria varied in length. The extracellular bacteria showed similar variation and were in close proximity to the monocytes. The staining reaction revealed characteristic single and bipolar condensation as described for Donovan bodies (Figure 16). The bacteria were pleomorphic with either bulging or tapered ends. The chromatin material stained intensely and was positioned centrally or at the periphery of the bacilli. The staining

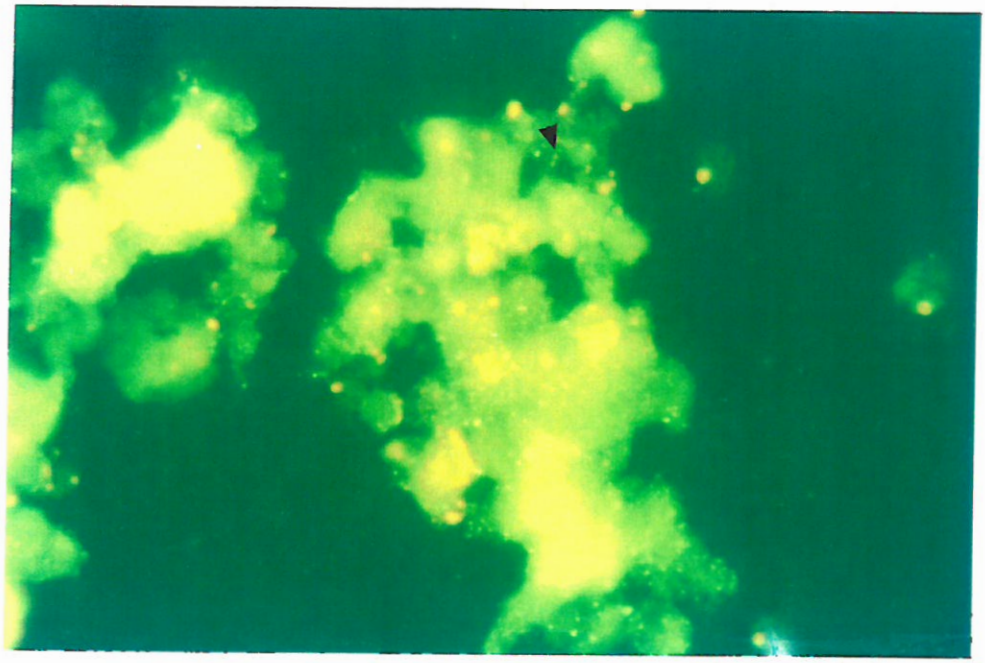


Figure 8: **Indirect immunofluorescence tests showing *Calymmatobacterium granulomatis* fluorescing brightly within infected mononuclear cells which had been reacted with sera from patients with granuloma inguinale (Mag x500).**

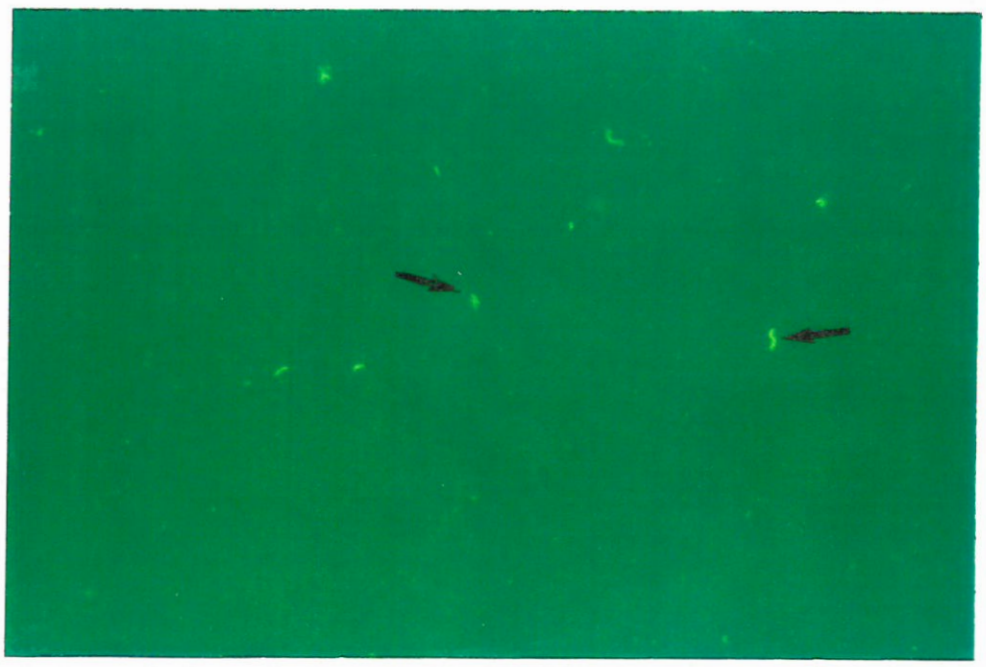


Figure 9: **Indirect immunofluorescence test showing brightly fluorescing individual bacilli (Mag x500).**

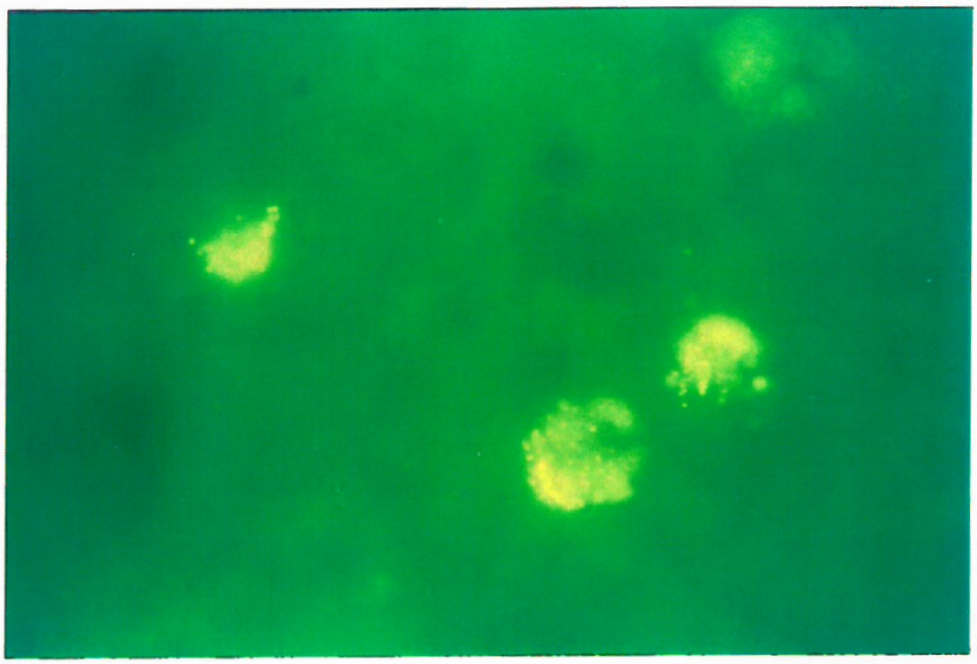


Figure 10: Indirect immunofluorescence test using sera from patients with granuloma inguinale showing intracellular fluorescing *Calymmatobacterium granulomatis* in smears obtained from lesions of granuloma inguinale (Mag x500).

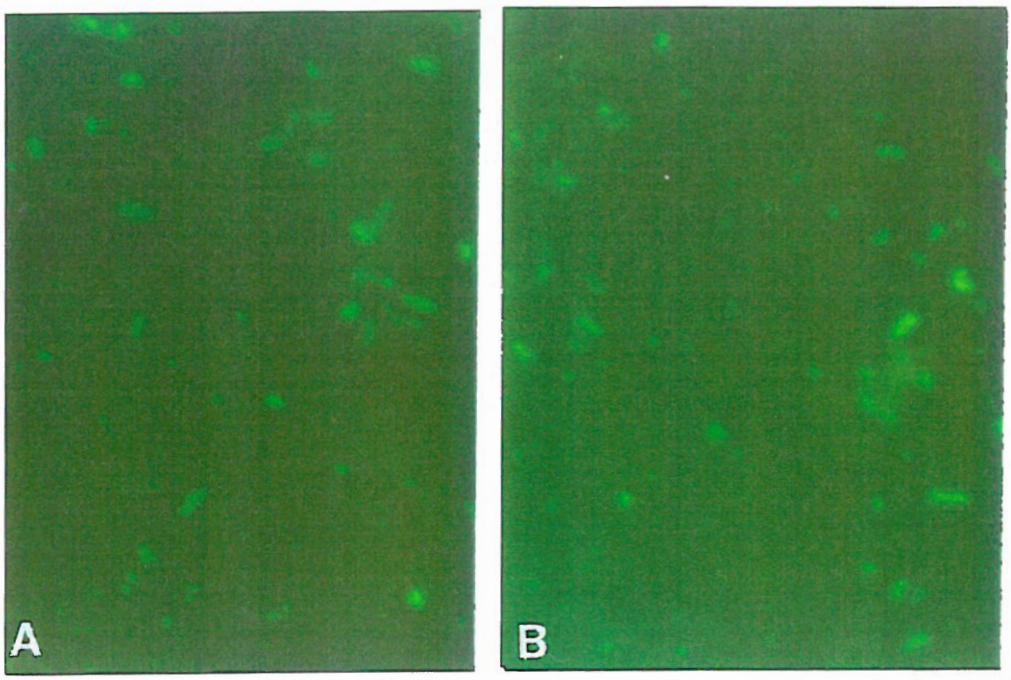


Figure 11: Indirect immunofluorescence showing absence of fluorescence of bacterial culture of *Klebsiella pneumoniae* using sera from patient with granuloma inguinale (A) and serum from normal human volunteer (B). Similar results were obtained for *K. oxytoca, K. aerogenes, Enterobacter cloacae, Citrobacter freundii* and *Escherichia coli* (Mag x500).

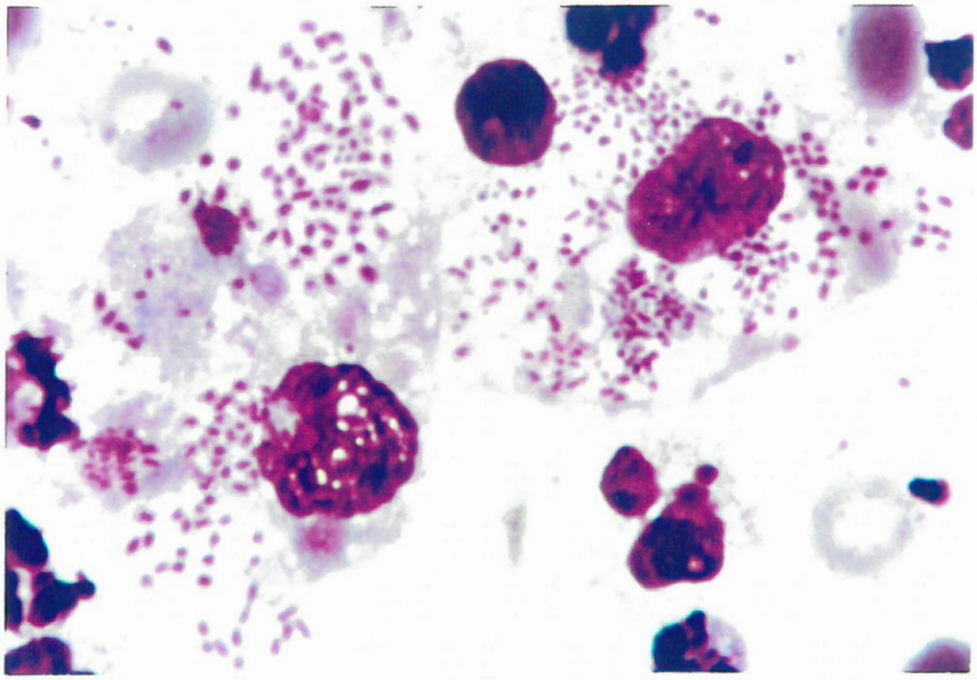


Figure 12: Light micrograph of an impression smear of granulation tissue of a genital lesion stained by RapiDiff showing large vacuolated mononuclear cells filled with numerous Donovan bodies in the cytoplasm (Mag x500).

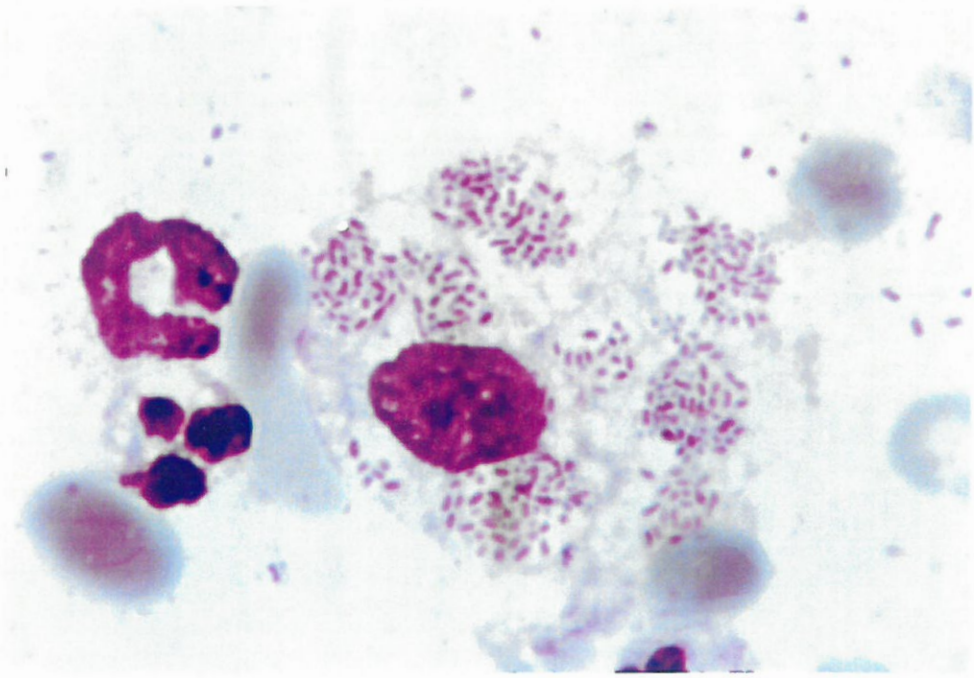


Figure 13: Light micrograph of an impression smear of granulation tissue stained by RapiDiff stain showing numerous Donovan bodies in pockets of vacuoles within the cytoplasm of the mononuclear cell (Mag x500).

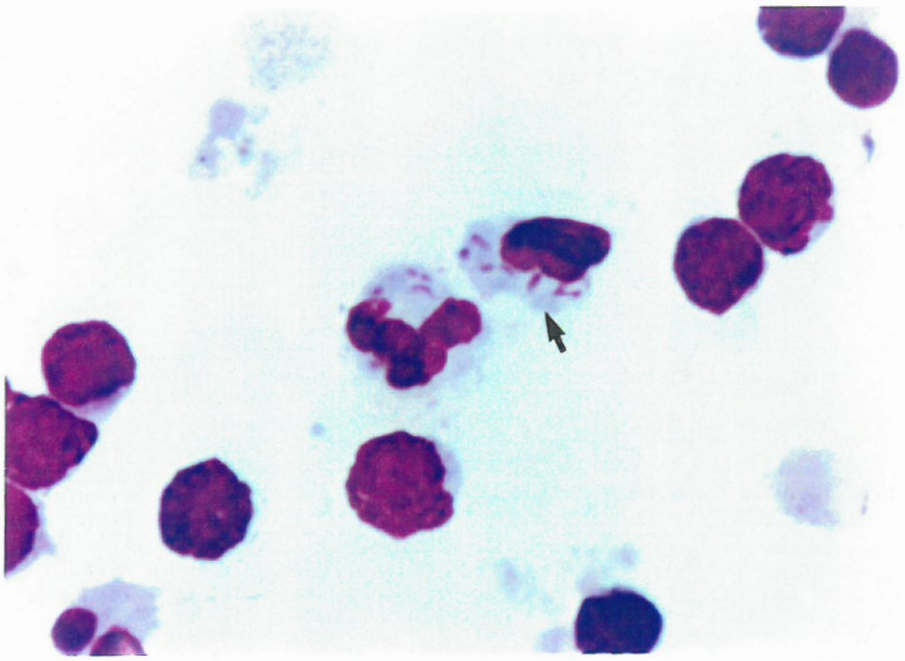


Figure 14: Light micrograph of RapiDiff stained preparations of monocyte co-cultures after 48 hours incubation showing infected monocytes with intracellular organisms (Mag x500).

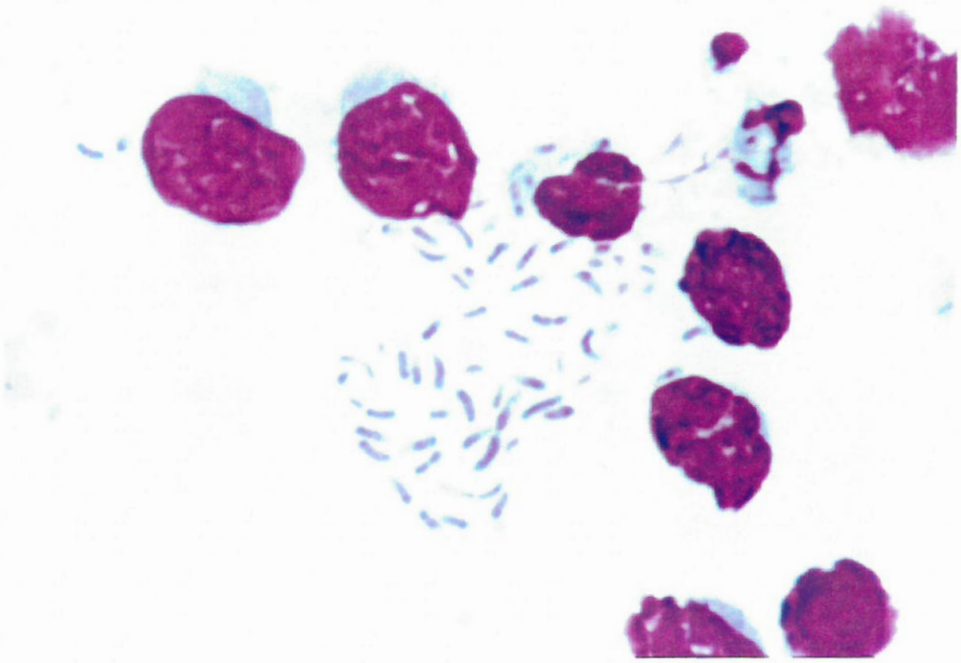


Figure 15: Light micrograph of RapiDiff stained preparations of monocyte co-cultures after 48 hours incubation showing infected monocytes with extracellular organisms (Mag x500).

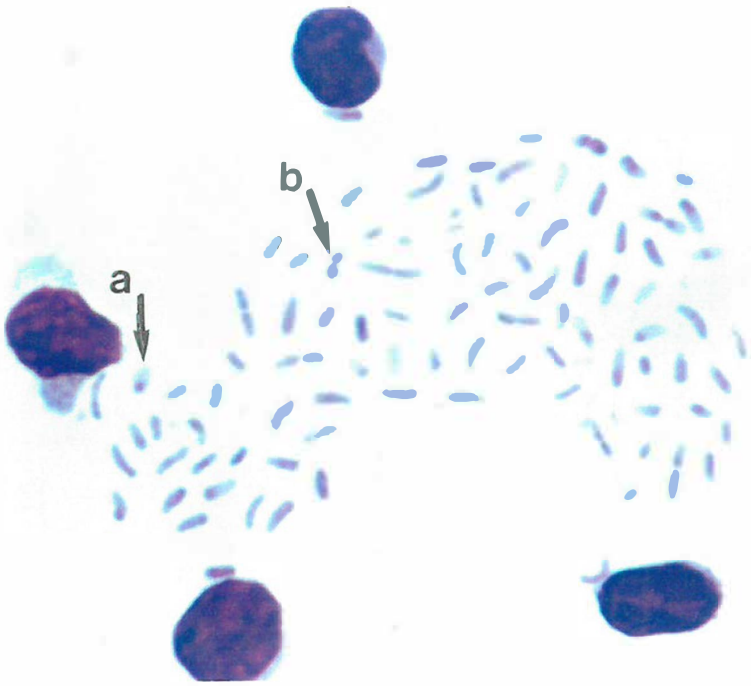


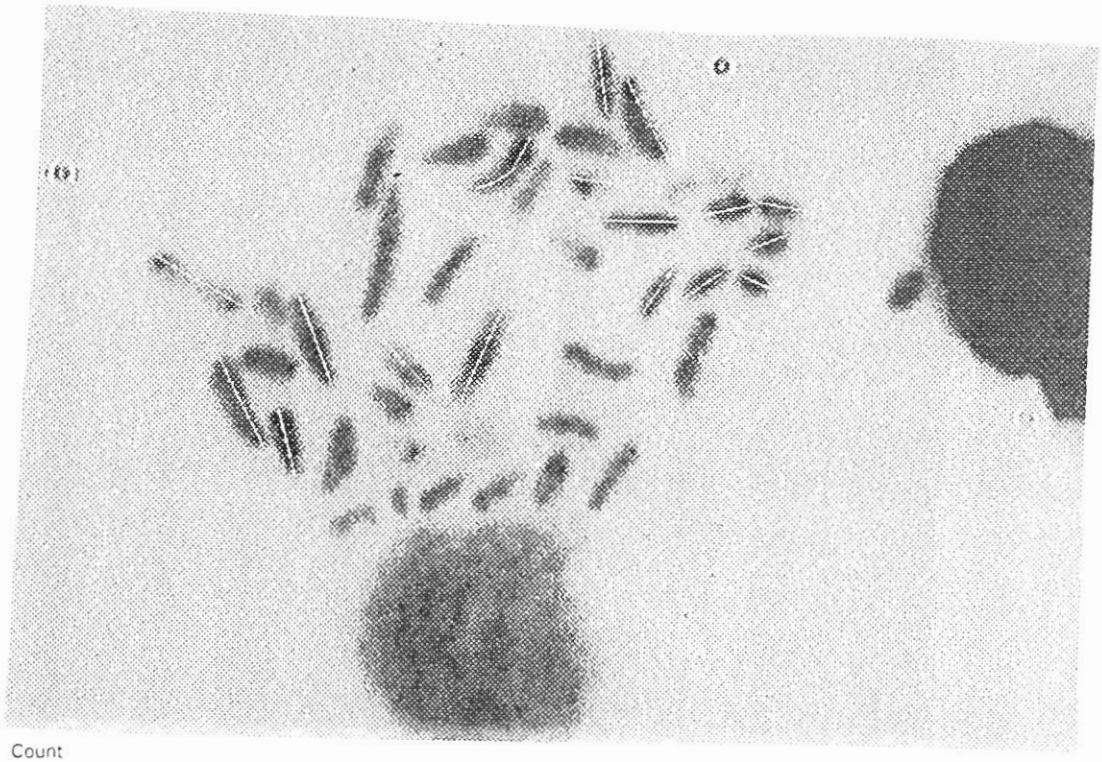
Figure 16: Light micrograph of RapiDiff stained preparations of monocyte co-cultures after 48 hours incubation showing infected monocytes with extracellular organisms displaying pleomorphism with distinct single (a) and bipolar (b) condensation (Mag x500).

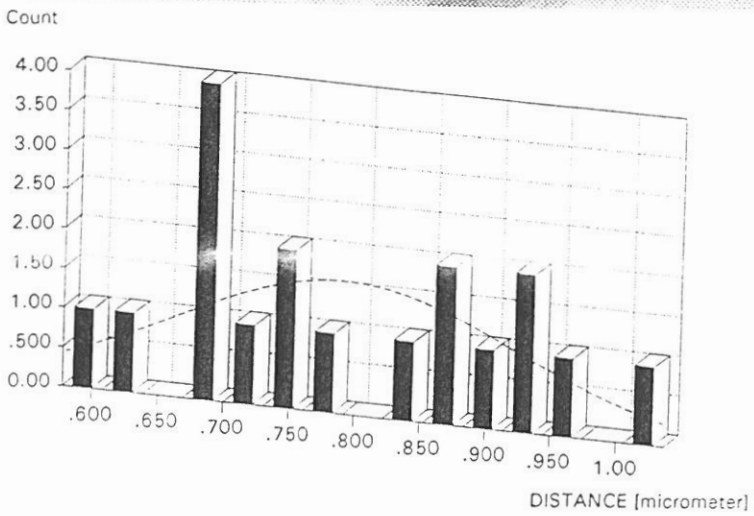
reaction of the bacilli was less intense as compared to the chromatin material. A large number of dividing bacilli were observed. These were elongated and comma shaped and appeared to divide into two unequal cells. The Gram stain showed the organism to be Gram negative with tapered ends (Figure 17). The size of the bacteria after the first 48 hours incubation ranged from 0,6μm to 1,0μm with a mean of 0,79μm in width and 0,9μm to 3,2μm with a mean of 2,0μm in length (Figure 18).

Subculture into fresh monocytes showed an increase in the number of bacterial cells. This increase was considerable at 48, 60 and 72 hours (Figures 19, 20 and 21). The morphology of these bacilli was predominantly short, plump and coccobacillary with considerable evidence of pleomorphism (Figure 19). A translucent area was also present around the cells. The single and bipolar condensation was still evident (Figures 20 and 21). The chromatin material was located centrally or to the periphery of the bacilli. Two distinct forms were present. The large elongated bacilli had a pale central area with intense staining of the bipolar ends giving the characteristic "safety pin" appearance. The coccobacillary form had an intense central area with a pale outline of the bacilli. Gram staining of the monocyte co-cultures showed the bacteria to be Gram negative (Figure 22). These were present as clusters displaying extreme pleomorphism. Some bacilli were either tapered or bulging with intense staining at the ends. The slender bacilli with the tapering ends were present more frequently in the 48 hour and 60 hour cultures than in the 72 hour culture. The size of the bacteria in the secondary culture ranged from 0,5μm to 1,0μm with a mean of 0,7μm in width and 0,8μm to 1,6μm with a mean of 1,2μm in length (Figure 23).

In later experiments monocyte derived macrophages (7 day old monocytes) were used for both primary infection and subsequent passaging. During the primary inoculations a large number of bacteria were seen intracellularly (Figure 24). The morphological characteristics of the intracellular bacteria was similar to those seen extracellularly. However, the morphology of the bacteria within the cytoplasm varied considerably and the nuclei of these cells had been displaced to the periphery of the cytoplasm as *in vivo*. In the secondary passage culture the coccobacillary forms were present intracellularly (Figure 25). These intracellular bacteria were present as a single bacillus or as a group

Figure 17: Light micrograph of Gram stained smear of 48 hour culture of infected monocytes showing Gram negative pleomorphic, elongated organisms with tapered ends (Mag x500).





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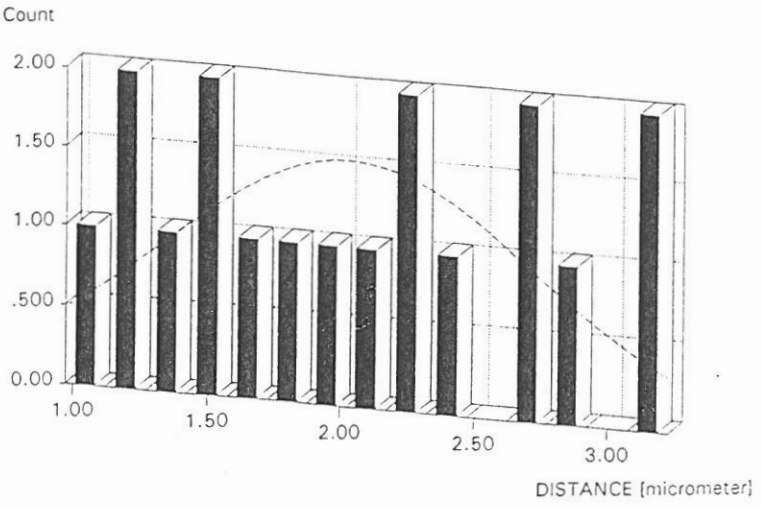
kurtosis	-1.05492



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kurtosis	-1.16614

Figure 18: Estimation of the size of the bacteria after the first 48 hours incubation. The size ranged from 0,6μm to 1,0μm with a mean of 0,79μm in width and 0,9μm to 3,2μm with a mean 2,03μm in length.

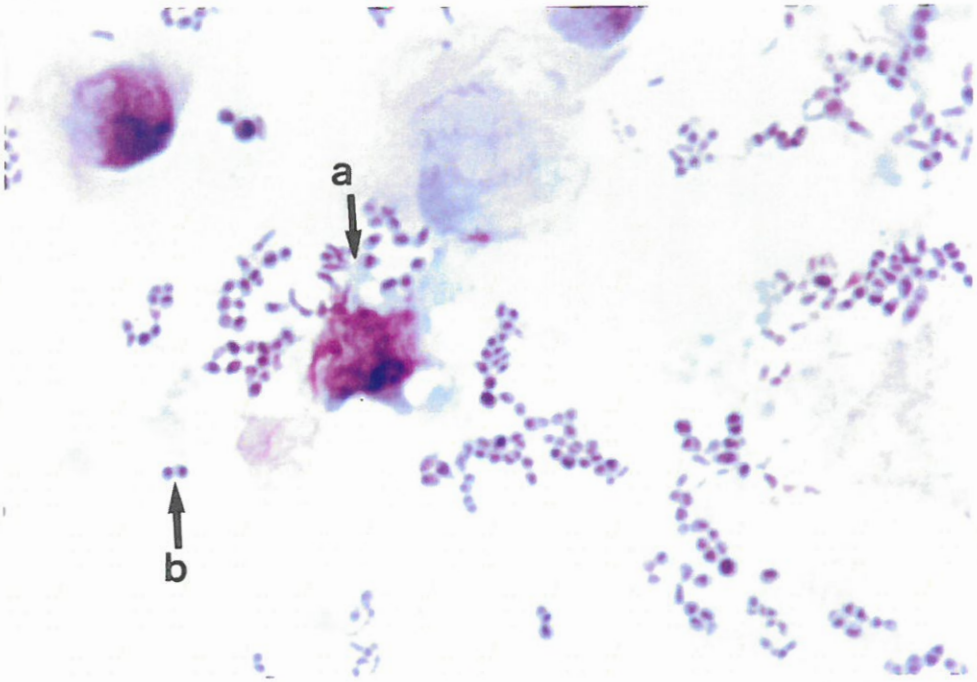


Figure 19: Light micrograph of RapiDiff stained smears of secondary inoculation of monocytes after 48 hours post infection showing an increase in the number of extracellular, coccobacillary forms with single (a) and bipolar (b) condensation (Mag x500).

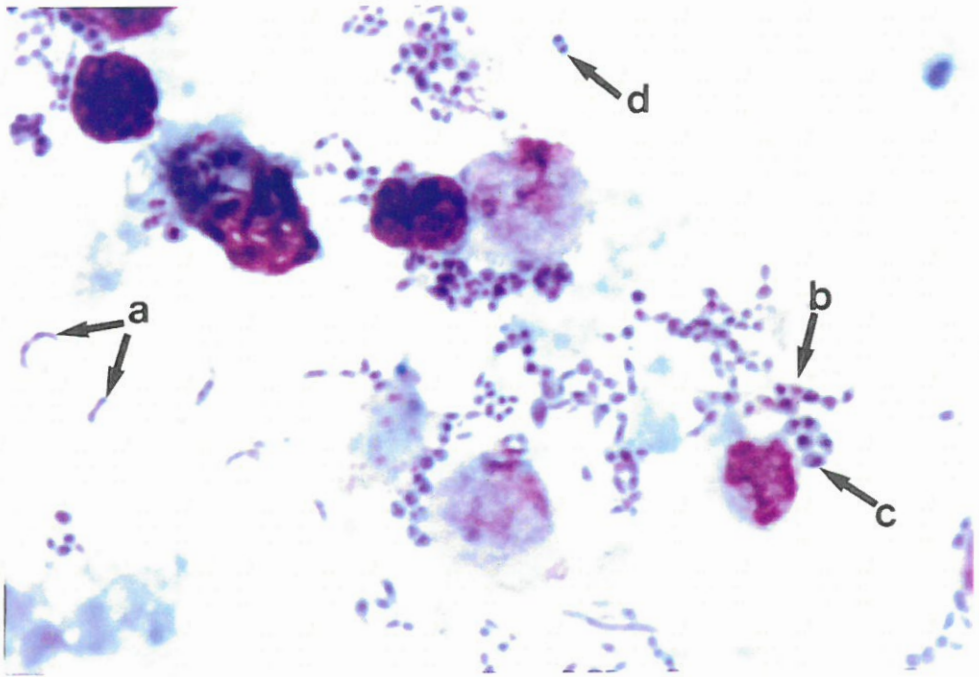


Figure 20: Light micrograph of RapiDiff stained smears of secondary inoculation of monocytes after 60 hours post infection showing an abundant growth of organisms which are extracellular, pleomorphic with tapering ends (a), coccobacillary forms (b) and single (c) and bipolar (d) condensation (Mag x500).

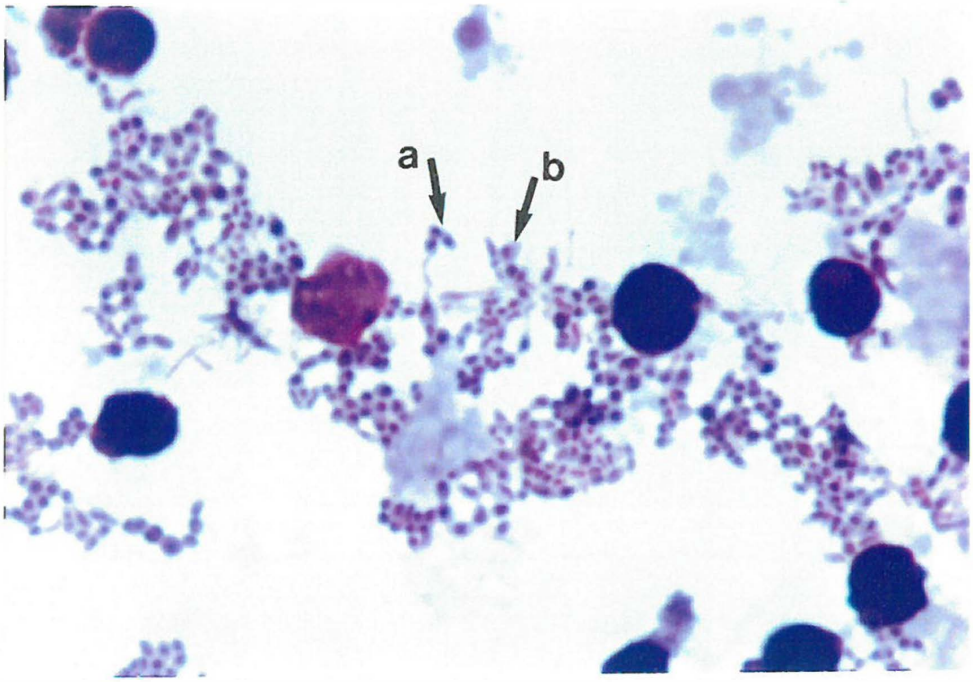


Figure 21: Light micrograph of RapiDiff stained smear of secondary inoculation of monocytes after 72 hours post infection. The culture had adapted to the medium. Note two forms of bacilli are evident. Large elongated bacilli with a pale central area and intense staining of the bipolar ends giving the characteristic "safety pin" appearance (a); coccobacillary forms with an intense central area and pale outline of the bacilli (b) (Mag x500).

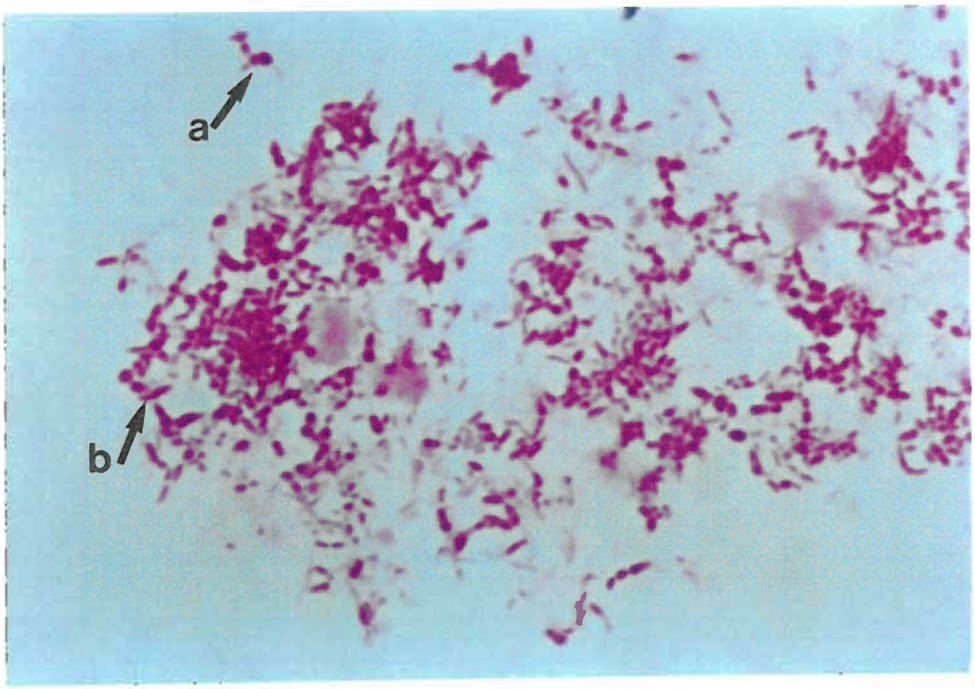
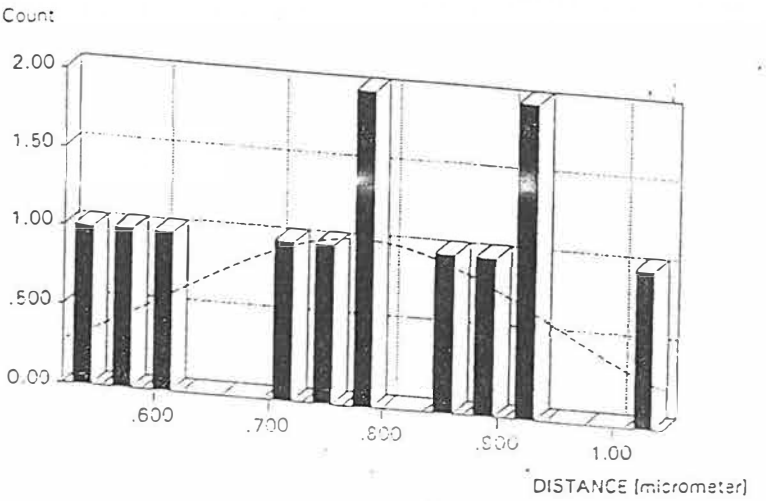


Figure 22: Gram stained smears of infected monocytes showing cluster of pleomorphic Gram negative bacilli with intense staining at the ends which are either bulging (a) or tapered (b) (Mag x500).



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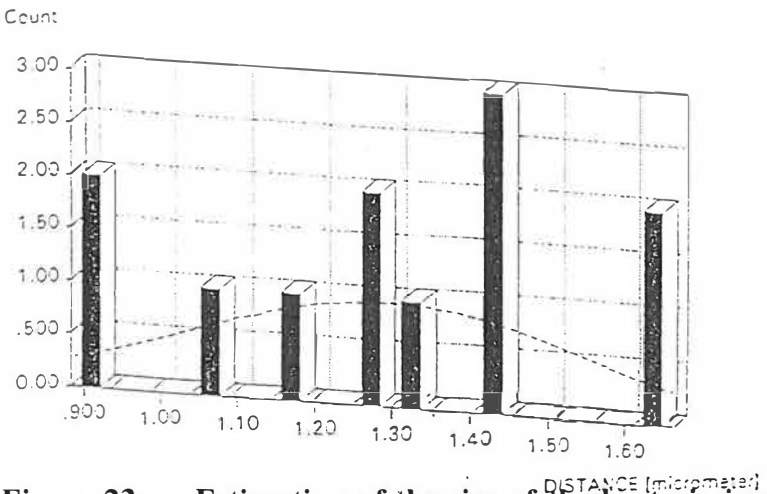

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kurtosis	-0.864323

Figure 23: Estimation of the size of the bacteria in the secondary culture. The size ranged from 0,5μm to 1,0μm with a mean of 0,7μm in width and 0,8μm to 1,6μm with a mean of 1,2μm in length.

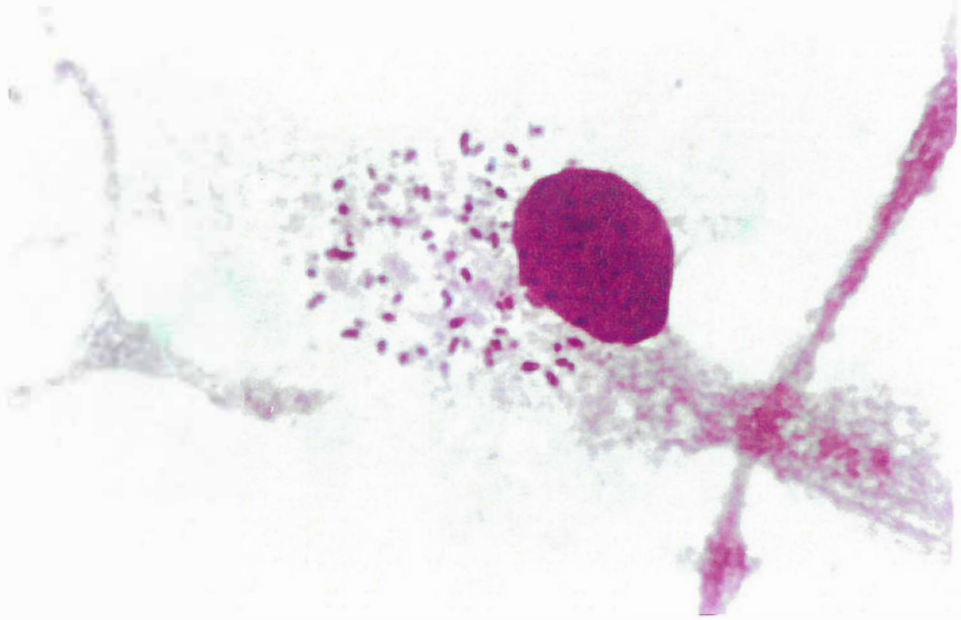

Figure 24: RapiDiff stained smear of a primary culture in monocytes showing intracellular bacteria. Individual bacilli are seen within vacuoles. The nucleus has been displaced to the periphery of the cell (Mag x500).

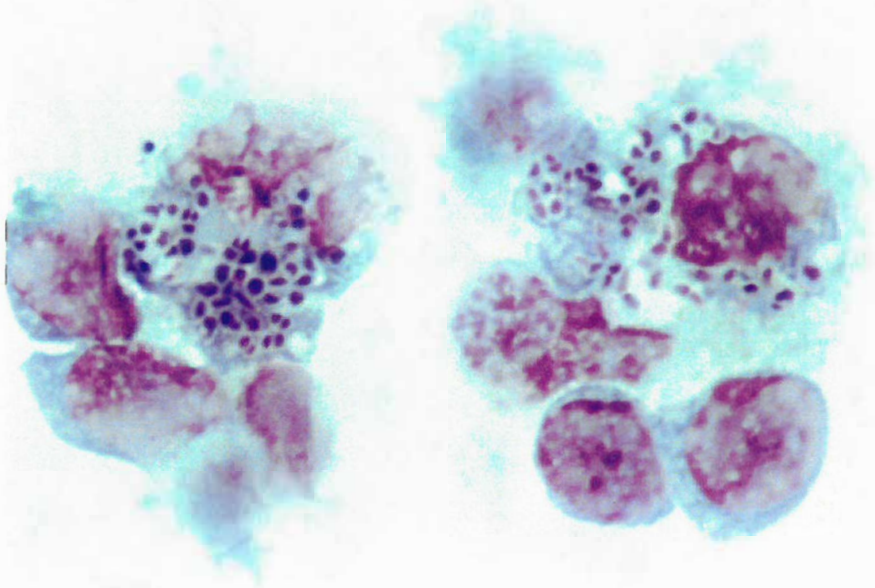

Figure 25: RapiDiff stained smear of a secondary culture in monocytes showing intracellular bacteria. Individual bacilli are seen in vacuoles or clusters of bacilli are seen within the vacuole. The nuclei have been displaced to the periphery of the cell (Mag x500).

of bacilli within a single vacuole. Some of these bacilli stained more intensely than others. The nuclei of these cells were also displaced to the periphery of the cytoplasm. Using light microscopy the capsules were not clearly defined. However, in certain areas of the coverslips these were seen more easily than in other areas (Figure 26). When the bacilli were seen in clusters, the capsules had coalesced to form a large halo around the bacteria. The haloes were smaller for some organisms as compared to others.

3.3.5 QUANTIFICATION OF *IN VITRO* GROWTH AND GROWTH CURVE OF *CALYMMATOBACTERIUM GRANULOMATIS*

At 24 hours post - infection, the organisms in the culture were approximately 10^3 organisms. The bacteria rapidly increased in number reaching a maximum around 72 hours post infection. The numbers declined after 96 hours. Cultures were counted at 24, 48, 72, 96, and 120 hours (Figure 27).

The growth curve showed a lag period for growth up to 24 hours. Small increases could be detected by 48 hours. The organisms in the infected monocyte co-cultures remained in exponential growth phase from day 3 to day 4; whilst some cultures continued to remain in the exponential phase by day 5. Although initially the generation time appeared to be slow, it was estimated to be 24 hours. The stationary phase of growth in cultures was relatively short; lasting for approximately 1 day, which resulted in a decrease in viable organisms. The bacteria from subcultures made on day 5 appeared to recover more slowly than those made at 48 hours.

3.3.6 *IN VITRO* EFFECT OF *CALYMMATOBACTERIUM GRANULOMATIS* ON HOST CELLS

The monocyte monolayers were infected with biopsy specimens after 20 hours of preparation. After the incubation period, the cells were activated showing large filopodia and vacuoles. Bacteria were seen extracellularly and in close association with the monocytes. In later experiments the monocyte monolayers were allowed to develop to the phagocytic macrophage stage ranging from day 5 to day 7 and thereafter inoculated

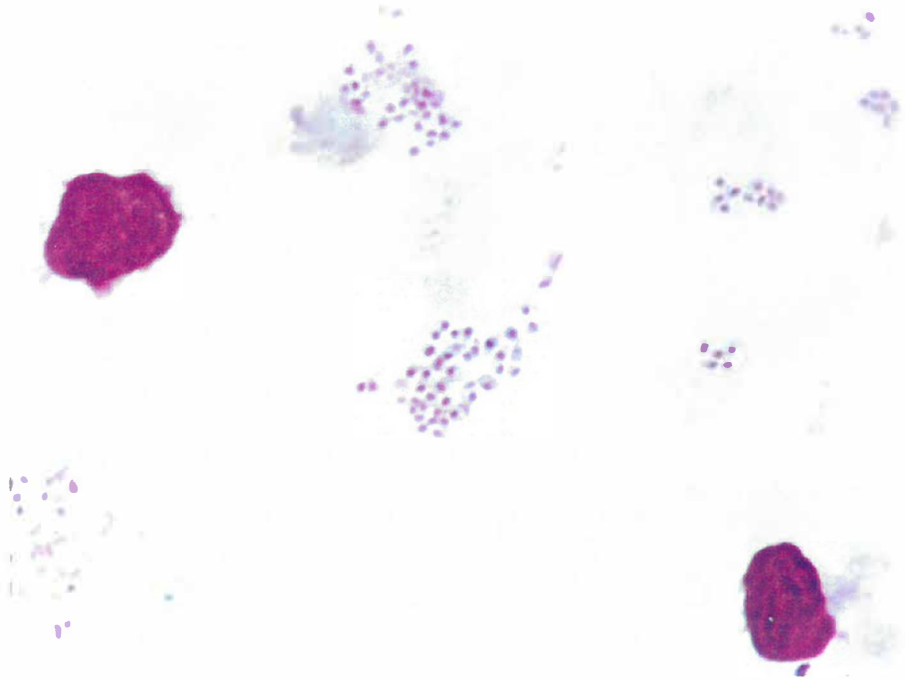

Figure 26: RapiDiff stained smear showing clearly defined capsules around each bacterium. In some areas the bacilli have aggregated and the capsules have coalesced to form a large halo around the cluster (Mag x500).

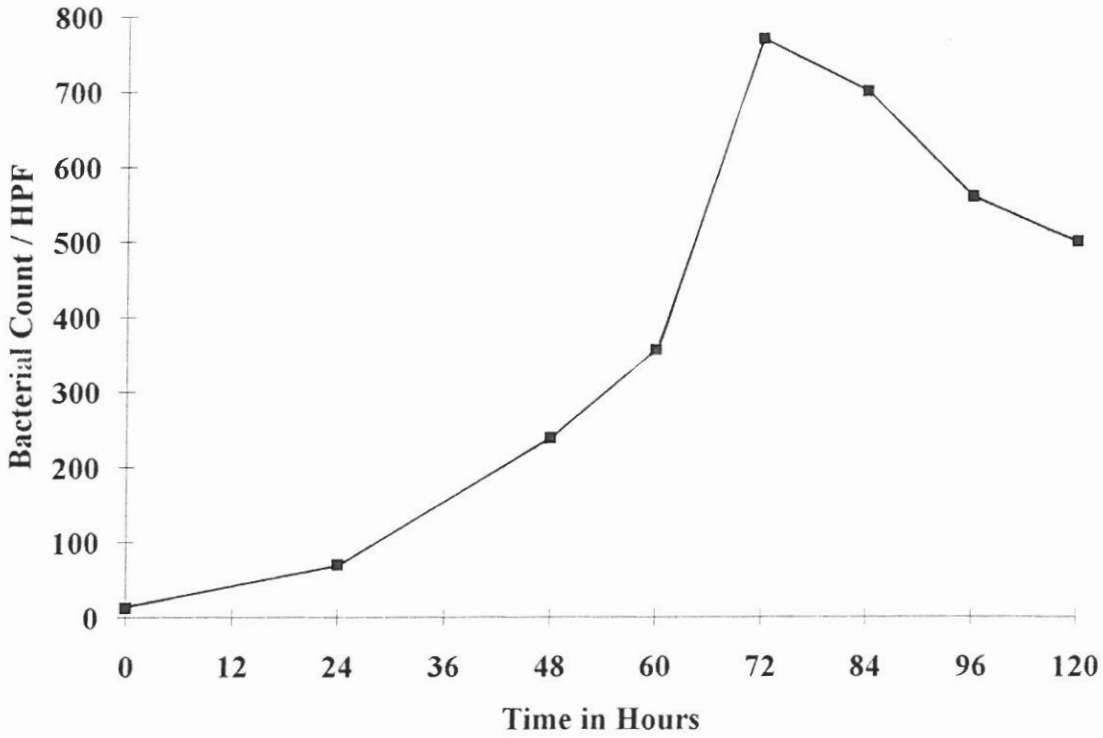

Figure 27: Growth curve of *Calymmatobacterium granulomatis* bacteria in monocyte co-cultures. The results were expressed as the mean of three experiments for each culture.

with biopsy specimen homogenate and tissue scrapings. After incubation the macrophages were highly activated and phagocytic. No pathological effects were observed since the cells were normal and had not detached from the coverslips (Figure 28).

3.3.7 TRANSMISSION ELECTRON MICROSCOPY

The bacteria in culture were seen predominantly extracellularly. The organism displayed a Gram negative cell wall structure with an electron dense capsule around the bacterial body. Some organisms were dividing extracellularly by pinching in of the cell wall and cell membrane. Details of the electron microscopy results are presented in chapter 4.0.

3.3.8 SEQUENCE ANALYSIS OF CULTURED ORGANISM

Bacterial DNA was amplified yielding a 1380 base pair product which was subjected to sequence analysis. The results of which are presented in chapter 5.

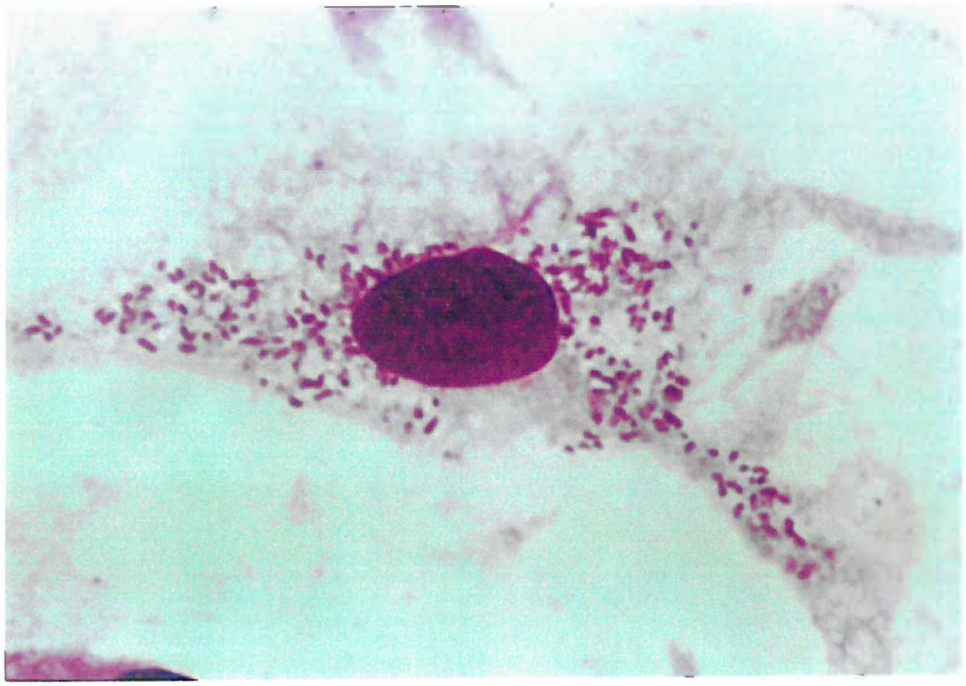

Figure 28: Growth of *Calymmatobacterium granulomatis* in 7 day old culture of monocytes. After 48 hours incubation intracellular bacteria were present within the cytoplasm. The monocyte is activated showing vacuolation. Cytopathic effect of the cell is not evident (Mag x500).

3.4 DISCUSSION

The *in vitro* cultivation of the aetiological agent of granuloma inguinale in the yolk sac of embryonated eggs (Anderson 1943; Anderson *et al*, 1945, Sheldon *et al*, 1945, Beveridge, 1946; Jennison *et al*, 1947; Dienst *et al*, 1948; Dulaney *et al*, 1948; Goldberg, 1959; 1962) and intracerebral inoculation in one day old chicks (Thomison, 1951) had been reported. Past attempts to cultivate the organism either as a primary or secondary culture on cell free media has been reported with limited success. The validity of previous reports has been questioned in view of the unavailability of the organism from any type culture collections. Initial efforts in this study were directed at attempting to repeat the work of the early researchers (1940 - 1960), but met with no success. The only success one could report on was on the maintenance of the organism after inoculation of the yolk sac of 5 - 7 day old embryonated eggs. There was no obvious growth and the attempts at subculture into further yolk sacs failed. Maddocks *et al*, (1976) also reported on persistent failure to culture the organism in the yolk sac of embryonated eggs.

The use of tissue culture for the *in vitro* cultivation and propagation of the aetiological agent of granuloma inguinale has not been described before. In this study, a technique for the successful propagation of the organism in human monocytes has been developed. Attempts were made to grow the organism in a monocyte cell culture in order to maintain a convenient system for *in vitro* study. The choice of peripheral blood monocytes for tissue culture was based on the visualisation of the causative agent within mononuclear cells of specimens (smears and biopsy) of infected tissue.

During the initial phase of the study, PBMNC were obtained from a single donor, a method which proved as expected to be fairly restrictive. The availability of cells from HIV negative blood donors from the Natal Blood Transfusion services facilitated the regular supply of monocytes. A natural extension of this work to ensure a continuous source of cells would be the use of a transformed monocyte cell line. The use of transformed cells would be convenient, since non transformed cells have a limited life time, however, using transformed clls also has its limitations because it is possible that

surface receptors expressed by the transformed cells differ from those of non-transfomed variety (Pinot *et al,* 1983).

A predictable problem of growing organisms from genital site lesions is that of contamination with other less fastidious organisms which would overgrow *C. granulomatis* and kill the monocytes. Initially, attempts were made to decontaminate these specimens by use of an aminoglycoside only. This group of antibiotics do not enter eukaryotic cells and are therefore expected to kill all susceptible extracellular bacteria. This would include all extracellular *C. granulomatis*, because aminoglycosides have been used for the successful treatment of granuloma inguinale (Maddocks *et al*, 1976; Richens, 1991; Hoosen *et al*, 1996). However, it was hoped that there would be sufficient numbers of intracellular bacteria to maintain growth of the organism. This approach was successful and the growth of *C. granulomatis* from biopsy specimens of three women with granuloma inguinale has been reported (Kharsany *et al*, 1996). However, in view of the persisting contamination of many biopsy specimens with aminoglycoside resistant Gram positive cocci and anaerobic bacteria, metronidazole and vancomycin were added on the presumption that *C. granulomatis* is not an anaerobic organism. The combination of vancomycin and metronidazole together with amikacin for decontamination was used on additional biopsy specimens and on scrapings of genital ulcers. Successful culture of *C. granulomatis* was obtained from an additional six biopsy specimens and in 14 of 200 men with genital ulcer disease from whom ulcer scrapings were collected. Evidence for the multiplication of *C. granulomatis* in the culture system was based on the following observation: the increase in the number of bacterial cells with each passage, the striking morphological similarities of the cultured *C. granulomatis* to those seen in direct smears by light and transmission electron microscopy, the isolates did not grow on an extensive variety of cell free culture media and the demonstration of a positive reaction in the immunofluorescence test using sera of patients with granuloma inguinale. Furthermore, the 16S rDNA sequence analysis described in chapter 5.0 supports the evidence of growth.

In direct smears the Donovan bodies are seen intracytoplasmically within macrophages and this is pathognomonic for granuloma inguinale (Greenblatt and Barfield, 1952).

The bacteria within these cells appear as either short coccobacillary or very pleomorphic encapsulated forms. It is suggested that the pleomorphic forms are undergoing a process of bacterial elongation and active replication. The pleomorphic nature of the organism in culture has been described by several workers (Anderson *et al*, 1945; Rake and Oskay, 1948; Goldberg, 1962), but it is unclear which study really reported on *C. granulomatis*. The bacteria in the monocyte co-culture after 48 hours incubation at 37°C in CO_2 (5%) in air were extremely pleomorphic, varying in size and shape in comparison to those usually seen in direct smears, suggesting that these organisms were in a process of cell division and multiplication.

The morphology of the bacteria varied after subsequent passages, either being pleomorphic or coccobacillary. During these subsequent passages the capsule and the single or bipolar staining reaction of the organisms were still evident. This morphology is attributable to viable cultures demonstrating the varying stages of growth suggestive of a highly sufficient growth environment. Some authors have reported the growth of what they believed to be *C. granulomatis* on cell free growth media (Anderson *et al*, 1945; Dulaney *et al*, 1948; Goldberg, 1959; Goldberg, 1962). Attempts were made to obtain growth in these media, however, despite the published methodology for the preparation of the media was carefully followed, there was no success. It is possible that unpublished details or unidentifiable differences in the ingredients may be the reason for these negative results. Another explanation could be that previously reported isolates of *C. granulomatis* which grew on cell free media belonged to a different species, most likely a contaminant or eg. *Haemophilus ducreyi*, which was not well described and not easy to culture at that time.

Capsules are often not visible by direct microscopy and the use of light microscopy for the interpretation of the presence or absence of capsules has been subjective. However, they can be observed by the use of special stains or as a halo around the cell when suspended in a dark colloidal substance such as India ink. In direct smears the capsules of *C. granulomatis* have been demonstrated with the Wright's stain as limited, dense, pinkish material surrounding a blue bacillary body with darker chromatin condensations, however a halo is sometimes seen around the organism (Anderson *et al*, 1945). This

suggests that with the Wright's stain the capsule does not always take up the stain. However, the bacteria may be encapsulated or unencapsulated. It is unclear what the exact reason might be for the capsule not taking on the stain. The capsule structure is not always seen by light microscopy. The capsules of the cultured bacteria stained with varying intensities with each passage. The reason for this is the adaptation of the bacteria to the medium which enhanced the extent of the capsular material. Transmission electron microscopy (Chapter 4) confirmed the presence of extensive capsules in the cultured bacteria.

Inoculation of the organism in monocyte derived macrophages, resulted in the parasitisation of the monocytes with no cytopathic effect. Histological examination of biopsy specimens also showed no evidence of cytopathic effect of the macrophages. Anderson *et al*, (1945a) found the Donovan organism to infect the epithelial cells of the yolk sac causing little injury to these epithelial cells. It has not been established as yet if *C. granulomatis* grown in cells other than monocytes/ macrophages would have a similar effect.

Donovan bodies were observed in the direct smears of specimens used for inoculating monocyte cultures, however the exact numbers of bacteria were unknown. Enumeration of viable bacteria has been problematic. Viable, non-culturable bacterial populations cannot be enumerated using conventional culture media, however the use of methods for detecting respiring cells, radiolabelled substrates uptake and differential staining techniques allow quantification by alternative methods (Rozak and Colwell, 1987). The definition of viability of procaryotes is not straightforward and this property is possessed by that portion of bacterial cells capable of multiplying when optimal conditions for growth are provided. The fact that there was an increase in the number of bacterial cells as observed by light microscopy in subsequent passages would suggest that these bacteria were viable and multiplying. Since the numbers of the bacteria per infected cell varied, it was not possible to accurately count the bacteria prior to inoculation of monocyte co-culture system. Therefore, the quantification of organisms in the experimental studies have been based upon direct microscopic visualisation and the counts of bacteria per high power field in stained preparations of the co-culture system.

It had not been possible to determine the absolute number of viable or infective organisms, since the passages were performed to obtain a high yield of cultured organisms. The growth curve of the organism obtained in this study showed direct evidence of multiplication of the organism in culture. Although the organisms were not estimated in the culture supernatant, the presence of 10^6 or more organisms / high power field at 60 to 72 hours post infection could be considered as a result from abundant growth of the organism. Such a yield of organisms is of great advantage for preparing infective material or specific antigens under controllable conditions for studies on the demonstration of pathogenicity and virulence.

In this study the bacteria grew both intracellularly and extracellularly. Those bacteria which multiplied extracellularly and in close proximity to the cells could be related to an essential growth factor being released from the cells. However, the early release of intracellular bacteria from the cells cannot be excluded. This suggests that the organism is a facultative and not a strict intracellular parasite. Anderson *et al*, (1945) also reported the presence of the organism in the yolk in an extracellular position and in the cytoplasm of the lining endodermal epithelium.

The presence of *C. granulomatis* specific antibodies in convalescent sera of patients with granuloma inguinale has been reported (Anderson *et al*, 1945). Similar responses have been observed when using *Klebsiella* antigen instead of *Calymmatobacterium* antigen (Rake, 1948; Packer and Goldberg, 1950). In this study the bacteria in the monocyte co-culture and direct smears showed bright fluorescence with sera from patients with granuloma inguinale and the absence of fluorescence with control bacteria. Serum from a volunteer who never had an episode of genital ulcer disease showed no reaction. Although there are controversies regarding cross reactivity with *Klebsiella* species in these tests, there was no reaction with the three species of *Klebsiella* used in this study. These results confirm the specificity of antibodies formed during infections with *C. granulomatis.*

The successful culture of the aetiological agent of granuloma inguinale will enable the collection of a sufficient number of bacterial strains to study the biological

characteristics and virulence attributes of this organism, to determine its antimicrobial susceptibility profile and to develop a definitive diagnostic test. This will lead to a better understanding of the epidemiology and pathogenesis of this disease.

CHAPTER 4.0
ELECTRON MICROSCOPIC STUDIES OF *CALYMMATOBACTERIUM GRANULOMATIS*

4.1 INTRODUCTION

Microscopy is the science of the interpretive use and application of microscopes. The aim is to form magnified images with the least optical defect and to achieve contrast between structures in the specimen under study. The resolving power of an objective lens differentiates microscopy into light and electron microscopy. Electron microscopy has made it possible to resolve cellular detail at the macromolecular level which is unachievable by light microscopy. The high resolution in electron microscopy is made possible by the use of a beam of electrons instead of light rays to produce the magnified image of an object. The role of electron microscopy in the elucidation of bacterial structure has been extensively reviewed (van Iterson, 1965; Costerton, 1979; Beveridge and Graham, 1991).

In granuloma inguinale the presence of bacillary forms intracellularly within macrophages of affected tissues has been observed consistently, and it is generally accepted that the disease results from this bacterial infection. *Calymmatobacterium granulomatis*, the causative agent of the disease, has not been cultured in bacteriological media and therefore the organism has been poorly studied. Histology of the granulation tissue shows the presence of macrophages, plasma cells, polymorphonuclear neutrophils and occasionally lymphocytes, multinucleated giant cells and dendritic cells. (Davis *et al*, 1969; Dodson *et al*, 1974; Kuberski *et al*, 1980). The macrophages within the diseased tissue are highly activated with numerous filopodia, increased amounts of lysosomes, rough endoplasmic reticulum, mitochondria, phagosomes and surface microvilli formation (Chandra *et al*, 1989). The bacteria are encased in vacuoles within the cytoplasm of cells, varying from one to 25 per cell. These vacuoles are likely to represent phagosomes or phagolysosomes.

Reports on its ultrastructural characteristics have been inconsistent (Davis *et al*, 1969; Davis, 1970; Dodson *et al,* 1974; Kuberski *et al*, 1980; Spagnolo *et al*, 1984; Chandra *et*

al, 1989). Earlier studies on the ultrastructure of *C. granulomatis* have been made possible by the use of adequately preserved tissue biopsy material, however, the preservation of the bacterial structure and morphology was poor (Spagnola *et al*, 1984). The effects of fixatives, dehydration and embedding procedures on the variation in the ultrastructural morphology of micro-organisms is well recognised (Silva and Sousa, 1973; Silva and Macedo, 1982).

Rake and Oskay (1948) vaguely described characteristics such as pleomorphism, "sticky" surface of the cell wall and polar granules within the cytoplasm. Although no definite capsules were seen, they concluded that on the basis of ultrastructural examination *C. granulomatis* and *Klebsiella pneumoniae* were morphologically similar. *C. granulomatis* has been recognised as a Gram negative organism by the presence of a characteristic trilaminar cell wall structure and the cell division by invagination of the cell wall and cytoplasmic membrane (Kuberski *et al*, 1980). The intracellular bacteria displayed were ovoid with a clear layer (electron lucent) surrounding the bacteria, the width of which varied considerably whilst for the extracellular forms this layer was larger and slightly more electron dense (Kuberski *et al*, 1980). These electron lucent areas have been interpreted as the bacterial capsules (Davis *et al*, 1969; Davis, 1970; Dodson *et al,* 1974; Kuberski *et al*, 1980; Spagnolo *et al*, 1984; Chandra *et al,* 1989). More recently Chandra and Jain (1991) have described the capsule as homogeneous material of varying density surrounding the organism. The electron dense granules within the bacterial body were prominent and generally found towards the periphery of the cell. The presence of bacteriophage either attached to the cell wall or as empty phage heads within the organism have been described (Davis *et al*, 1969; Davis, 1974). However, other workers have reported the absence of these (Dodson *et al*, 1974; Kuberski *et al*, 1980; Chandra *et al*, 1989; Chandra and Jain, 1991) on the basis that paracrystalline aggregates were absent. These aggregates are usually found during intracytoplasmic development of virus, hence does not support the hypotheses of bacteriophage replication within the organisms (Kuberski *et al*, 1980) and therefore the absence of bacteriophage.

The presence of pili and vesicles evaginating from the cell membrane (Dodson *et al*, 1974; Chandra, *et al*, 1989) have been reported. According to Chandra *et al*, (1989) these

vesicles could represent phage heads as described previously (Davis *et al*, 1969). The ultrastructural characteristics of the bacterial cell wall and cell membrane and the pinching in of these structures during cell division suggest that these organisms are Gram negative (von Iterson, 1969; Costerton, 1979) .

The aims of this study were to

- determine the ultrastructural morphology of *C. granulomatis* in culture
- determine the ultrastructural morphology and relationship of the organisms within the infected tissue
- compare the ultrastructural morphology of *C. granulomatis* in culture and in infected tissue.

4.2 MATERIALS AND METHODS

4.2.1 PROCESSING OF MONOCYTE CO-CULTURES

Cells from one monolayer of three different monocyte co-cultures infected with specimens of different patients (Chapter 3.0) were scraped off the coverslips with sterile pasteur pipettes.

4.2.1.1 Method

Each of the cell suspensions were centrifuged at 12 000 rpm for fifteen minutes and the deposits fixed in 1% glutaraldehyde in Eagles minimal essential media with Earle's balanced salts solution (EMEM) (Whitakker M.A. Bioproducts, Maryland, USA) for one hour and processed as outlined in table IX. The specimens were dehydrated through ascending grades of alcohol and embedded in an araldite epoxy resin (Glauert, 1956).

4.2.2 PROCESSING OF TISSUE BIOPSY SPECIMENS

Tissue biopsy specimens were obtained from three different patients with large exuberant, granulomatous lesions of more than one month duration and impression smears stained by Rapidiff showed Donovan bodies. Routine histological examination performed on these biopsies showed granulation tissue with marked acanthosis and pseudoepitheliomatous hyperplasia. A mixed inflammatory infiltrate and neutrophilic microabscesses were present. Histiocytes containing Donovan bodies were identified and the histological features confirmed a diagnosis of granuloma inguinale.

4.2.2.1 Method

The tissue biopsy specimens for electron microscopy were immediately placed in Karnovsky's fixative (pH 7,4) (Karnovsky, 1965) for 1 hour at 4° C. The specimens were diced into 1 mm cubes, re-immersed into fresh Karnovsky's fixative for a further

TABLE IX: PROCESSING SCHEDULE OF INFECTED MONOCYTE CO-CULTURES FOR ELECTRON MICROSCOPY

Step	Process	Solutions	Temp	Time
1	Fixation	1% glutaraldehyde in EMEM	24° C	1 hour
2	Wash	EMEM	24° C	2 min
3	Wash	EMEM	24° C	2 min
4	Fixation / Contrast	1% osmium tetroxide in EMEM	4° C	30 min
5	Wash	0,2M sodium cacodylate buffer	24° C	2 min
6	Wash	0,2M sodium cacodylate buffer	24° C	2 min
7	Dehydration	30% ethanol	24° C	10 min
8	Dehydration	50% ethanol	24° C	10 min
9	Dehydration	70% ethanol	24° C	10 min
10	Dehydration	90% ethanol	24° C	10 min
11	Dehydration	100% ethanol	24° C	10 min
12	Dehydration	100% ethanol	24° C	10 min
13	Dehydration	100% ethanol	24° C	10 min
14	Infiltration	Araldite + absolute ethanol 50 : 50	24° C	15 min
15	Infiltration	Araldite 1	60° C	1 hour
16	Embedding	Araldite 2	60° C	24 - 48 hours

30 minutes and processed using techniques outlined in table X. Specimens were embedded and polymerised in araldite epoxy resin (Gluaert *et al*, 1956).

4.2.3 ULTRAMICROTOMY

Semi-thin sections (1μm) were cut with a Reichert Ultracut ultramictrome using glass knives. Sections were collected onto glass slides, heat fixed, stained with 1% alkaline toluidine blue and examined with an Olympus BX40 photomicroscope. Fields of interest were selected and located on the block face which was trimmed to produce a "mesa" with a trapezoidal shape. Ultrathin sections (50 - 60nm) were cut, collected onto uncoated 200 mesh copper grids and double stained with uranyl acetate and Reynold's lead citrate for 2 and 3 minutes respectively (Reynold, 1963). If Donovan bodies were present as in figure 29, the blocks were processed for electron microscopy.

4.2.4 ELECTRON MICROSCOPY

Sections were viewed on the Jeol 100C transmission electron microscope at an accelerating voltage of 60 - 80 kV. Images were photographed using fine grain plate film.

TABLE X: PROCESSING SCHEDULE OF TISSUE BIOPSY SPECIMENS FOR ELECTRON MICROSCOPY

Step	Process	Solutions	Temperature	Time
1	Fixation	Karnovsky's fixative	4°C	1 hour
2	Wash	0,2 M sodium cacodylate buffer	24°C	10 min
3	Wash	0,2 M sodium cacodylate buffer	24°C	10 min
4	Fixation/Contrast	1% osmium tetroxide	4°C	1 hour
5	Wash	0,2 M sodium cacodylate buffer	24°C	10 min
6	Wash	0,2 M sodium cacodylate buffer	24°C	10 min
7	Dehydration	70% ethanol	24°C	30 min
8	Dehydration	90% ethanol	24°C	30 min
9	Dehydration	100% ethanol	24°C	30 min
10	Dehydration	100% ethanol	24°C	30 min
11	Dehydration	100% ethanol	24°C	30 min
12	Intermediate solvent	propylene oxide	24°C	30 min
13	Infiltration	propylene oxide : araldite (1:1)	24°C	30 min
14	Infiltration	Araldite	60°C	1 hour
15	Infiltration	Araldite	60°C	1 hour
16	Embedding	Araldite (Beem capsules)	60°C	24 - 48 hours

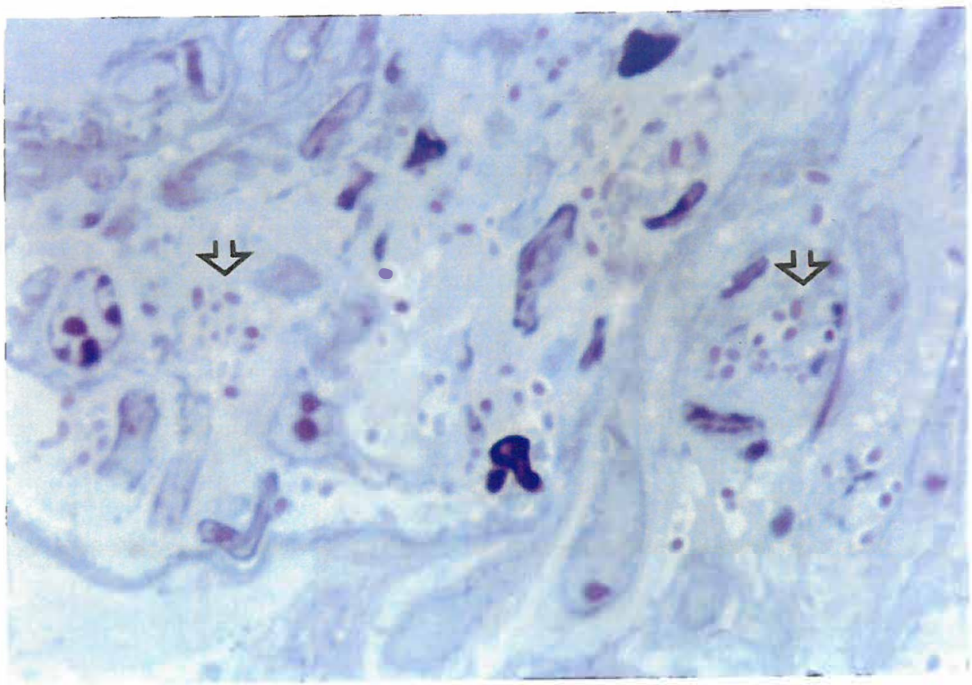

Figure 29: Light micrograph of alkaline toluidine blue stained tissue sections showing fields selected for electron microscopy (arrow) (Mag x500).

4.3 RESULTS

4.3.1 MONOCYTE CO-CULTURES

4.3.1.1 Ultrastructure of cultured *Calymmatobacterium granulomatis*

Transmission electron microscopy of the monocyte co-cultures showed the bacteria appearing extracellularly either in clusters (Figure 30) or singly (Figure 31). The bacterial cells displayed a dense homogenous capsule. Some of the organisms displayed pleomorphism (Figure 31). The bacteria were not observed within membrane bound vacuoles.

4.3.1.2 Morphology of cultured *Calymmatobacterium granulomatis*

The cytoplasm was rich in ribosomes many of them occurring in aggregates (Figures 32 and 33). Electron dense granules were prominent (Figures 32 and 33) and located to the periphery of the cytoplasm. The bacterial cells exhibited a characteristic Gram negative cell wall structure (Figure 34), consisting of an outer membrane, a middle electron opaque layer and an inner plasma membrane. The periplasmic space between the outer and inner membrane was electron lucent. Both plasma and outer membrane were approximately 0,007μm thick, each displaying the typical trilaminar nature (Figure 34). The capsule was thick, dense and fuzzy (Figures 32, 33 and 34). A trilaminar membrane was observed on the periphery of the capsule (Figure 34), whilst no other cell surface structures suggestive of phage particles, pili or flagella were identifiable.

4.3.1.3 Multiplication of cultured *Calymmatobacterium granulomatis*

The bacteria were seen to divide by binary fission into two unequal sized daughter cells. This was seen by pinching in of both the cell wall and cell membrane with septum formation (Figure 35). The capsule was evident as thick bundles of material with relatively high electron contrast.

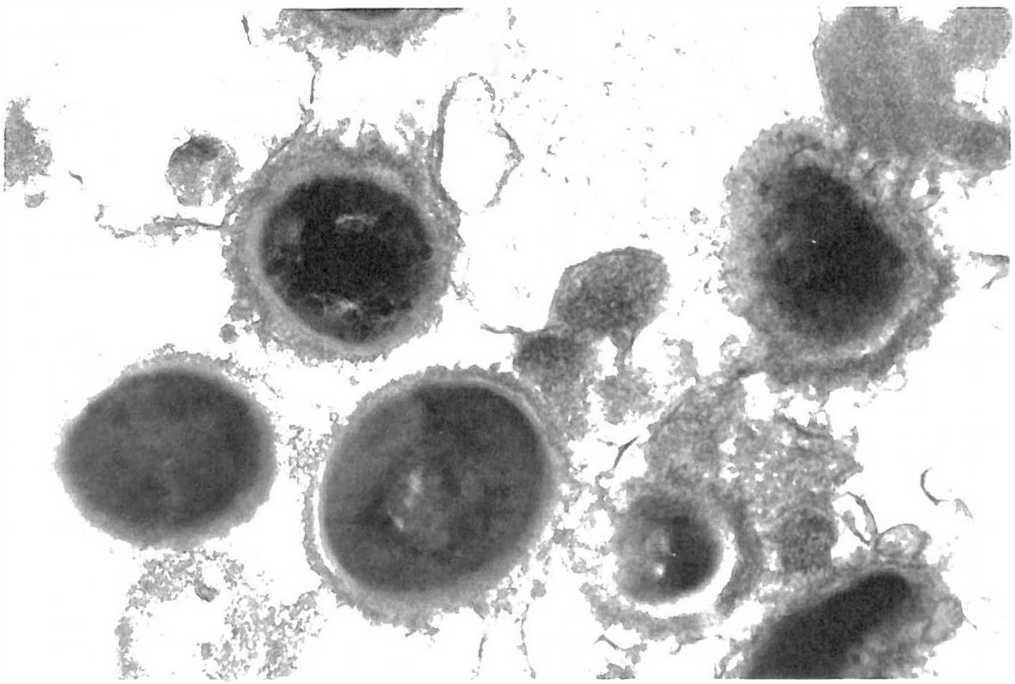

Figure 30: Electron micrograph showing a large number of extracellular organisms in clusters (Mag x36 000).

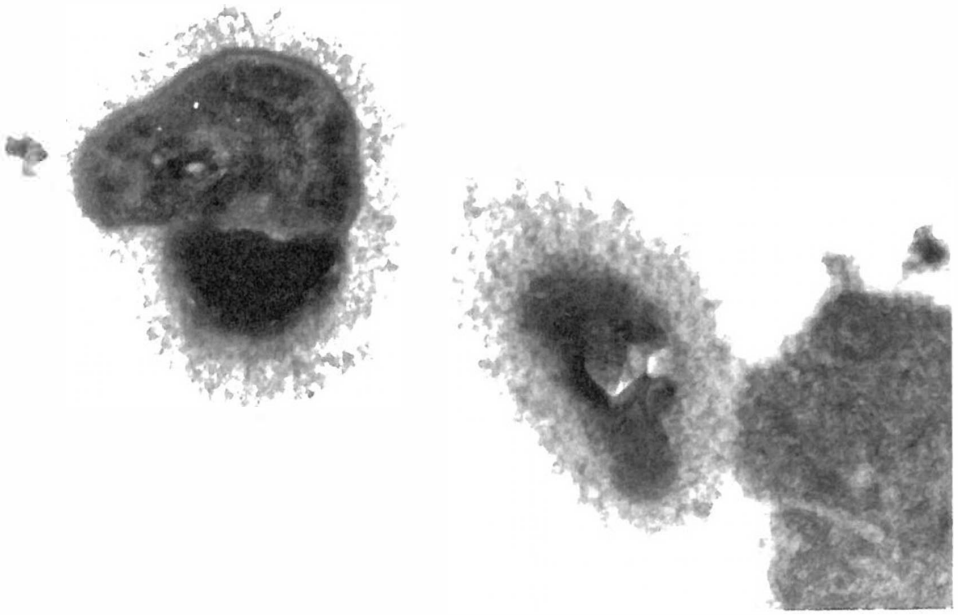

Figure 31: Electron micrograph showing extracellular organisms occurring singly and displaying pleomorphism (Mag x36 000).

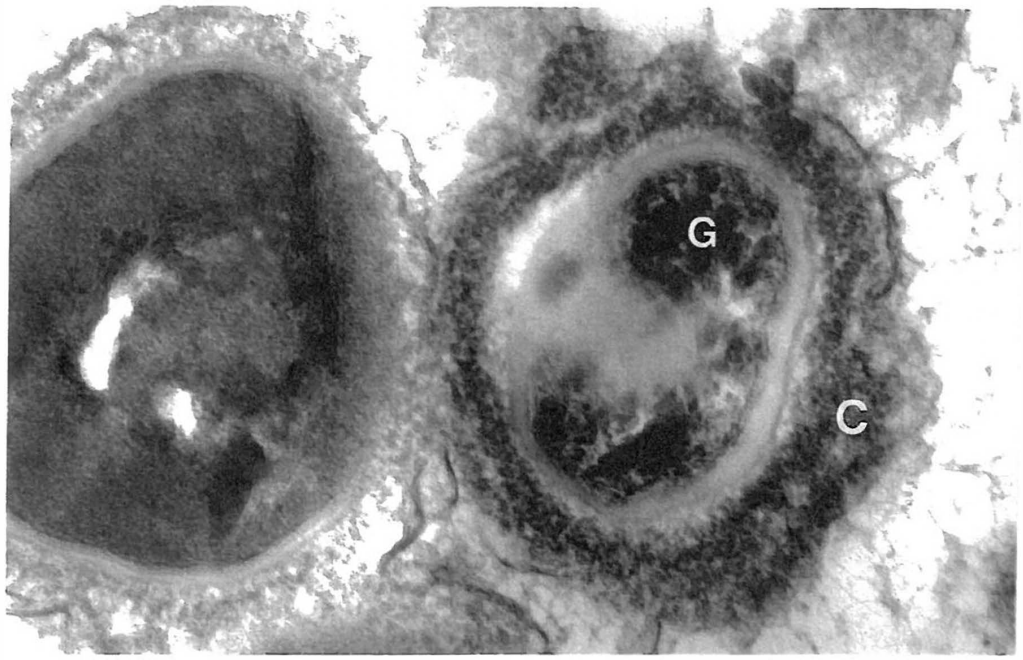

Figure 32: Electron micrograph showing the thick electron dense capsule (C) and numerous electron dense granules (G) within the cytoplasm (Mag x85 000).

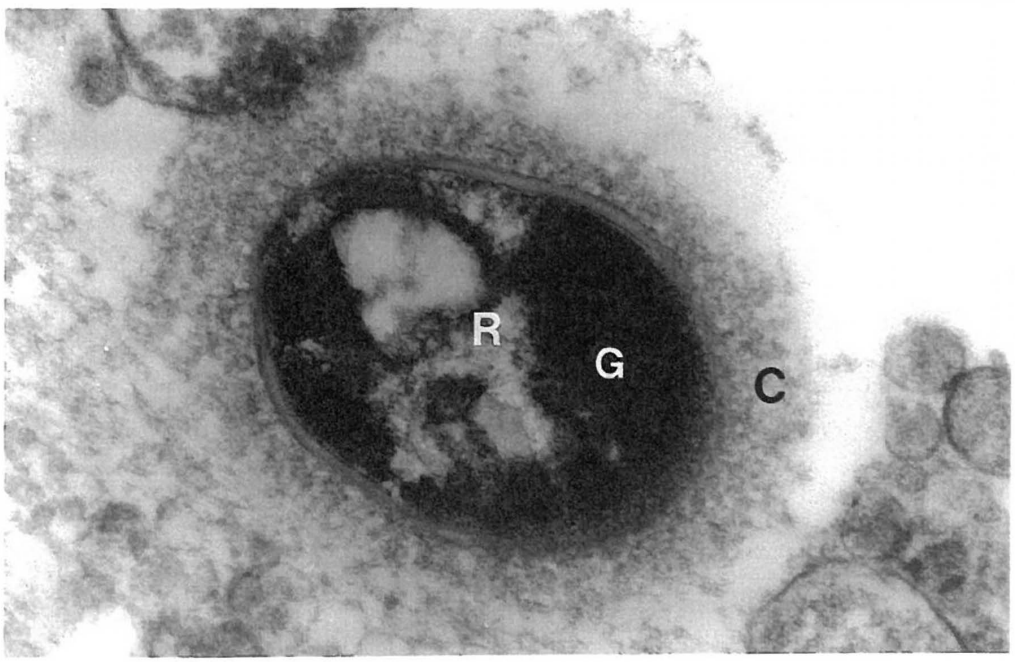

Figure 33: Electron micrograph showing the thick fibrillar electron dense capsule (C) with numerous electron dense granules (G) and ribosomes (R) within the cytoplasm (Mag x85 000).

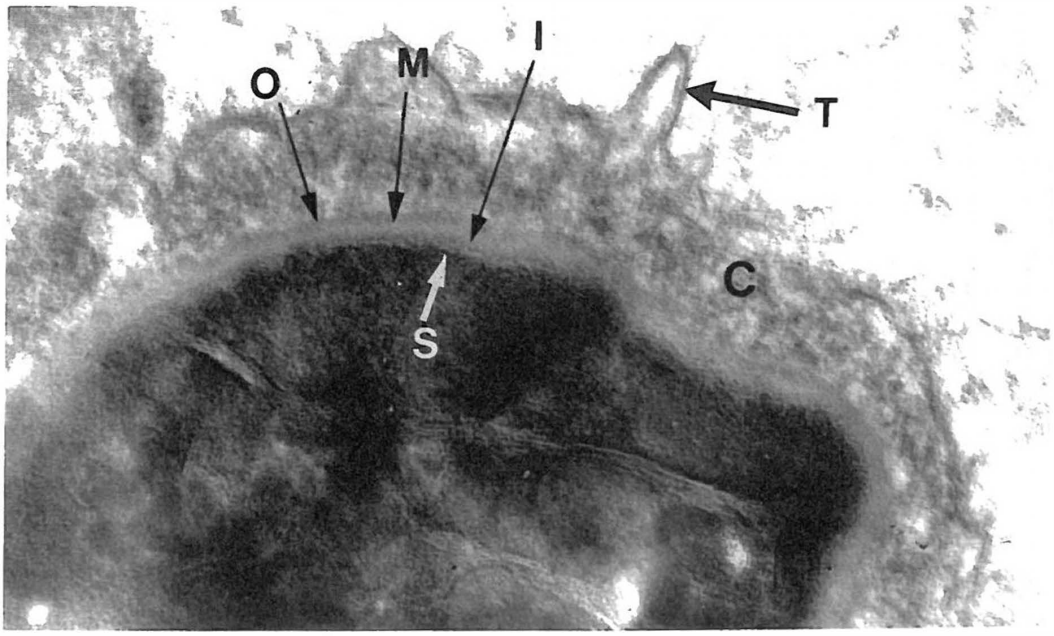

Figure 34: Electron micrograph displaying the Gram negative cell wall structure of the organism consisting of an outer membrane (O), middle electron opaque layer (M), inner plasma membrane (I) with periplasmic space (S) and a trilaminar membrane (T) around the capsule (C) (Mag x100 000).

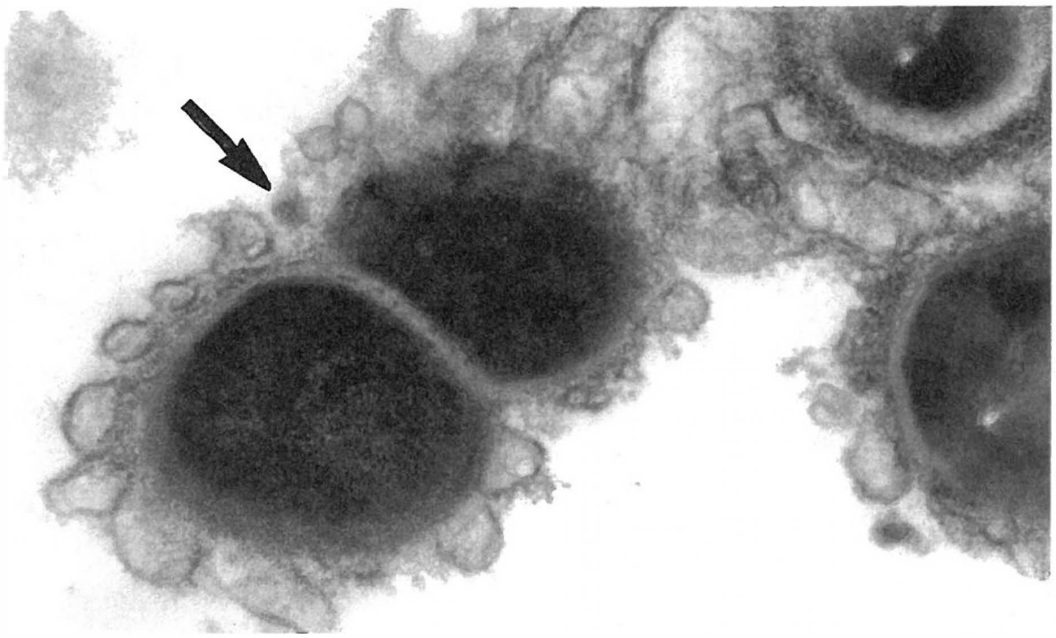

Figure 35: Electron micrograph showing an extracellular organism undergoing cell division. The capsule is prominent with an irregular outline (Mag x59 000).

4.3.1.4 Ultrastructure of lysed cultured bacteria

Among the bacilli occurring free in the monocyte co-culture, a few displayed ultrastructural signs of lysis with clearing of the intracellular compartment. In the lysed cells, the cell wall was clearly discernable as a trilaminar structure surrounded by an electron dense capsule (Figure 36). However, the capsule was narrower (Figure 36) than that of viable bacteria (Figures 32, 33 and 34).

4.3.2 TISSUE BIOPSY SPECIMENS

4.3.2.1 General features of the lesions

4.3.2.1.1 *Epidermal*

At low power magnification the epidermal layer appeared normal. The keratinocytes exhibited normal morphology. Large polyhedral shaped "prickle cells" characteristic of stratum spinosum were observed (Figure 37). Adjacent cells were bound by tight junctions namely desmosomes which had normal dense plaque and filaments. Bundles of tonofilaments were abundant intracellularly. Oedema was noted both intra and intercellularly. Mitochondria with sparse disrupted cristae were often observed. The epidermis depicted a focal inflammatory infiltrate which consisted predominantly of macrophages, plasma cells and some neutrophils (Figure 38). The plasma cells with lamellated cisternae of endoplasmic reticulum and lysosomes were evident.

4.3.2.1.2 *Dermal*

The dermis was infiltrated with a rich inflammatory exudate consisting predominantly of large macrophages, plasma cells, neutrophils and lymphocytes. The non infected macrophages had well developed endoplasmic reticulum, were rich in mitochondria and lysosomes and did not show any degree of necrosis. There was an increase in the number of plasma cells, which showed an abundance of well developed Golgi apparatus and rough endoplasmic reticulum. The nuclei of the infected phagocytic cells were often displaced

Figure 36: Electron micrograph showing a lysed bacterium with a clear intracellular compartment and distinct trilaminar cell wall structure. The density and thickness of the capsule is decreased (Mag x30 000).

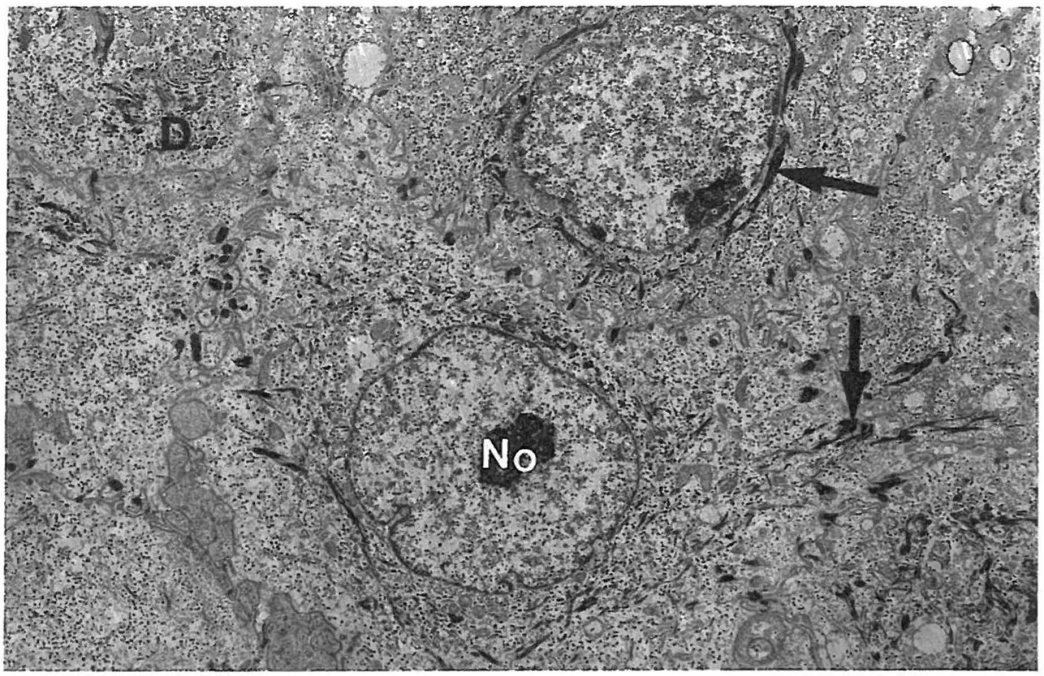

Figure 37: Low power electron micrograph showing the stratum spinosum of the epidermis. Adjacent cells are bound by desmosomes (D). Nucleoli (No) are prominent. Intracellular bundles of tonofilaments (arrows) are abundant (Mag x2 500).

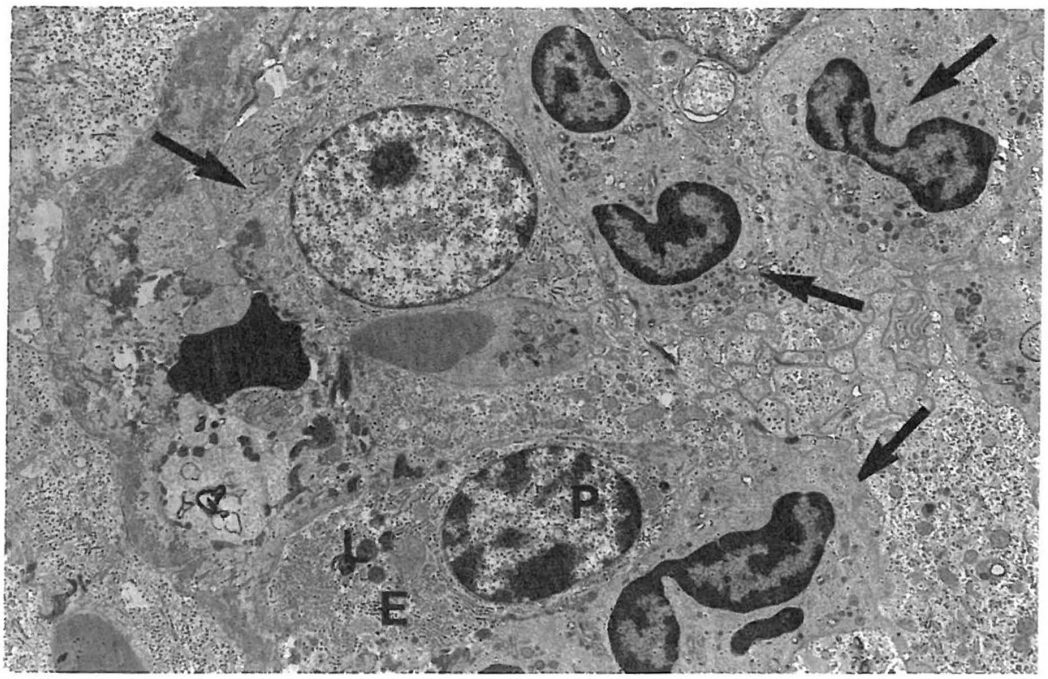

Figure 38: Low power electron micrograph depicting focal inflammatory infiltrate (arrows) within the epidermis. Note plasma cells (P) with lamellated cisternae of endoplasmic reticulum (E) and lysosomes (L) (Mag x2 500).

towards the periphery and the phagocytic vacuole containing the bacteria, occupied most of the cytoplasm. The cytoplasm of the infected cells were rich in mitochondria, rough and smooth endoplasmic reticulum and numerous Golgi complexes .

4.3.2.2 Location of bacterial cells

The bacteria were seen within the macrophages which had infiltrated the epidermal layer (Figures 39 and 40) and occasionally were seen lying in the intercellular spaces. In the dermal layer the bacteria were seen predominantly within histiocytes (Figure 41). These bacteria were localised either in isolated vacuoles (Figures 39 and 41) or as clusters of bacilli within a single vacuole (Figures 41). Occasionally the bacteria were found within vacuoles of necrotic neutrophils (Figures 42). The number of ingested bacteria varied considerably from 3 to 20 per cell.

4.3.2.3 Association of bacteria with phagocytic cell

There was considerable variation in the morphologic relationship of the phagocytic vacuole and the bacteria. The boundaries of each vacuole containing bacteria were either membrane limited or the vacuoles had broken membranes (Figure 43) or were lying in dense amorphus granular capsular material dispersed in the cytoplasm without a vacuole and limiting membrane of the vacuole (Figure 44). The degree of the denseness of the amorphous granular capsular material was variable and not discernible, at times being totally absent resulting in an electron lucent area (Figures 43 and 44).

4.3.2.4 Morphological characteristics of bacteria (Donovan body)

The shape and size of the bacteria in a single plane varied from being round, oval to elliptical in shape. They measured 1,0μm in width and 2,0μm in length (Figures 43 and 44). Whilst most bacteria were observed intracellularly, a few were seen extracellularly within intercellular spaces of the surrounding stroma (Figure 45). At a higher magnification the filamentous skein of centrally located nucleoplasm was evident and the nuclear material consisted of a less dense homogenous mass (Figure 46). The electron

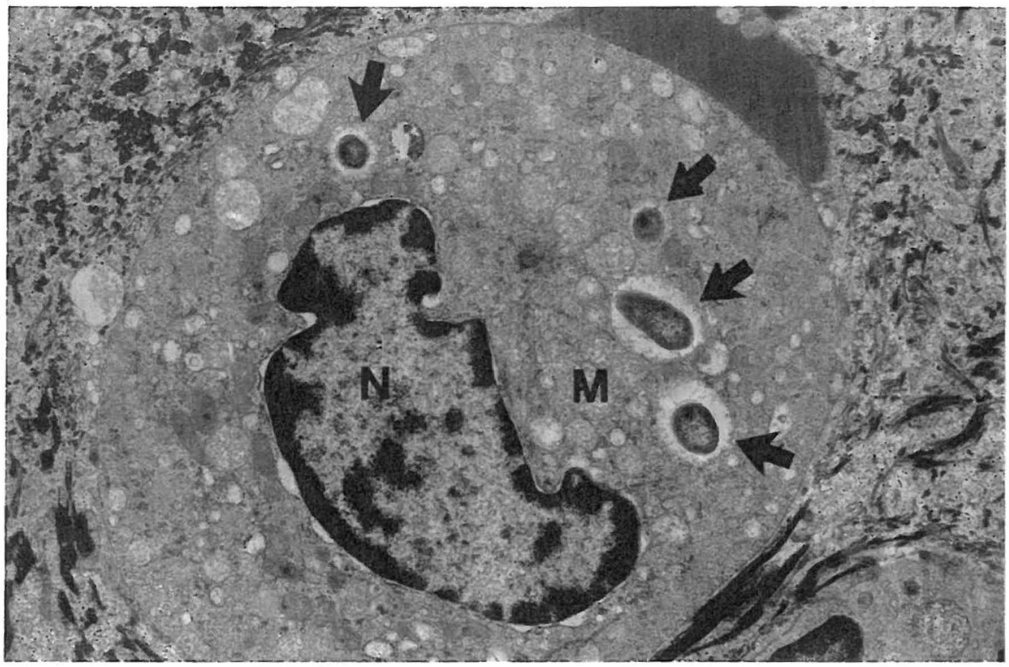

Figure 39: Electron micrograph showing a macrophage infiltrating the epidermis. Note a large nucleus (N), mitochondria (M) and cytoplasm with several phagosomes, each containing single bacterium (arrows) (Mag x5 000).

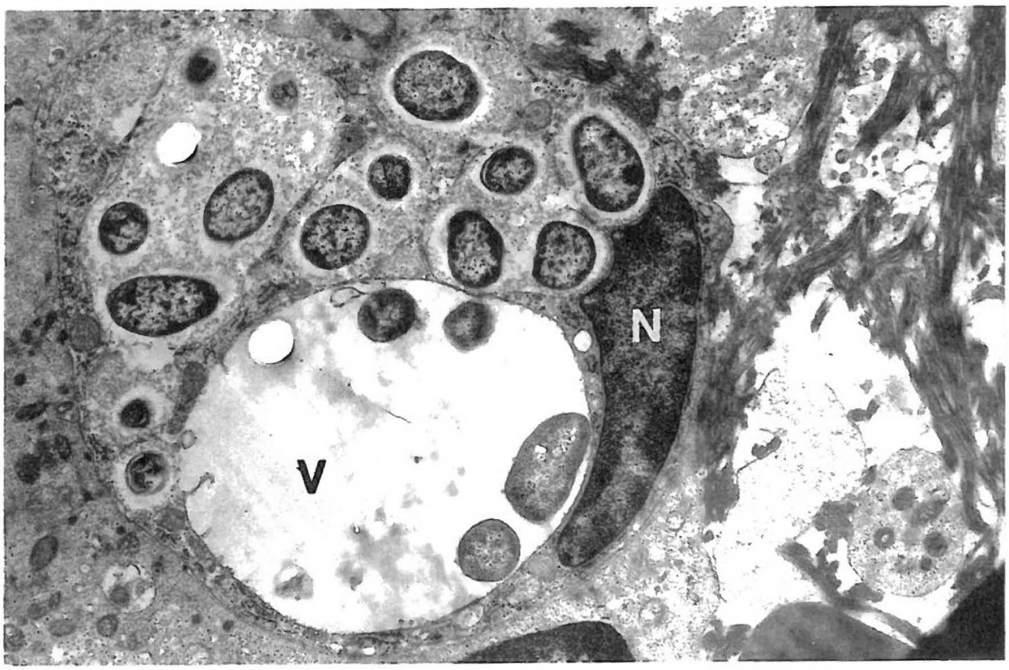

Figure 40: Electron micrograph showing the macrophage in the epidermis with intracellular bacteria. Note some are within a vacuole (V) bound by a vacuolar membrane, whilst others are lying free within the cytoplasm. The nucleus (N) has been displaced to the periphery of the cell by a phagocytic vacuole containing bacteria which occupies most of the cytoplasm (Mag x6 000).

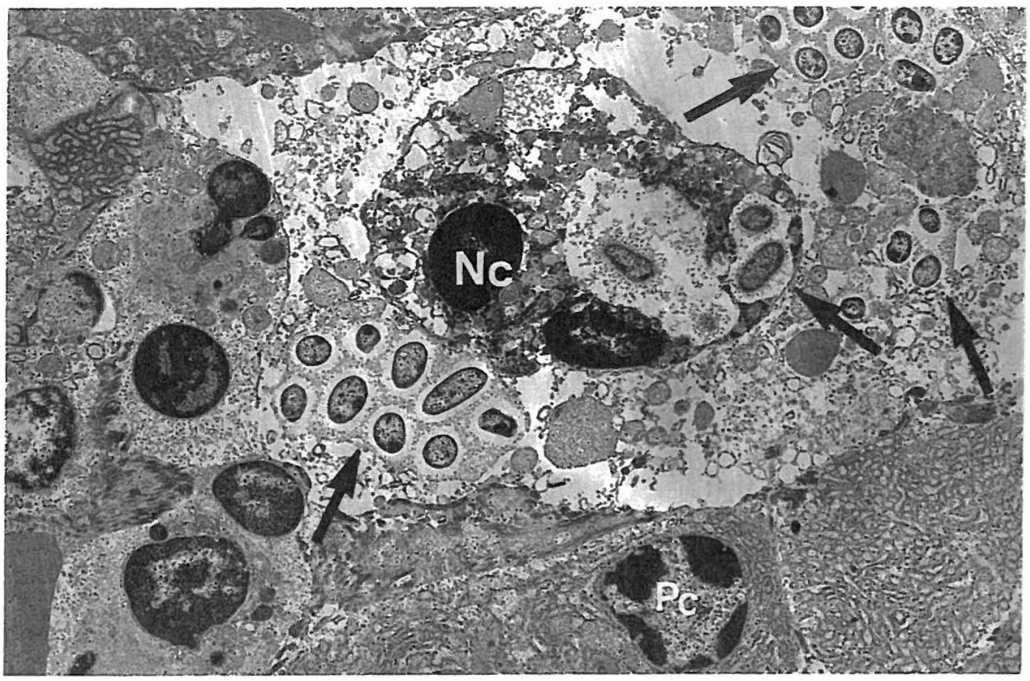

Figure 41: Low power electron micrograph showing a neutrophil (Nc) with ingested bacteria lying over a necrotic histiocyte. Bacilli are seen singly or aggregated in pockets (arrows). Adjacent to the histiocyte are a number of plasma cells (Pc) (Mag x3 000).

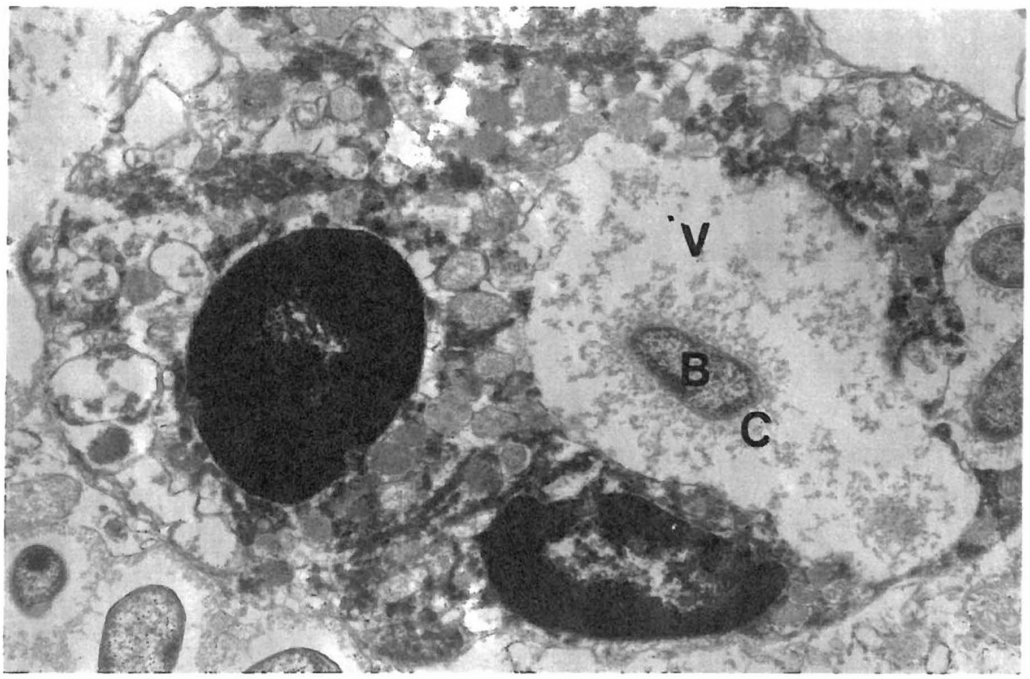

Figure 42: High power electron micrograph of figure 41 depicting a partially necrotic neutrophil with ingested bacteria (B) within vacuole (V). Capsular material (C) is dispersed throughout the vacuolar space (Mag x8 000).

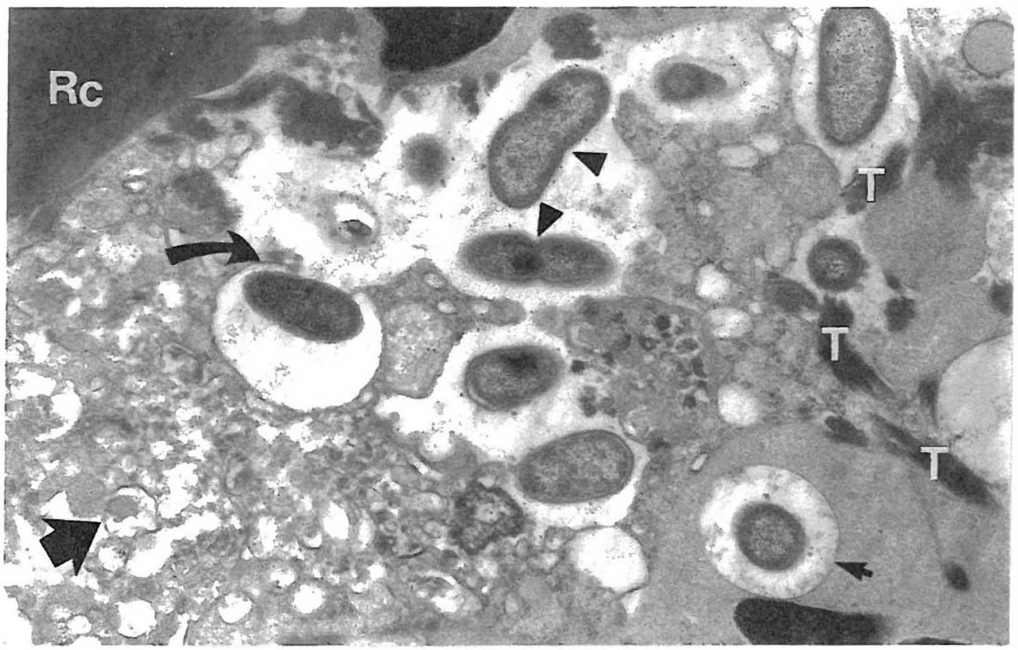

Figure 43: Electron micrograph depicting a highly vacuolated bacterial infected degenerate cell (▲). Note bacilli occur within phagosomal vacuoles bound by a limiting membrane (▲) or a broken membrane (▲) with some bacilli (▲) undergoing cell division. Red blood cells (Rc) and tonofilaments (T) are present (Mag x8 000).

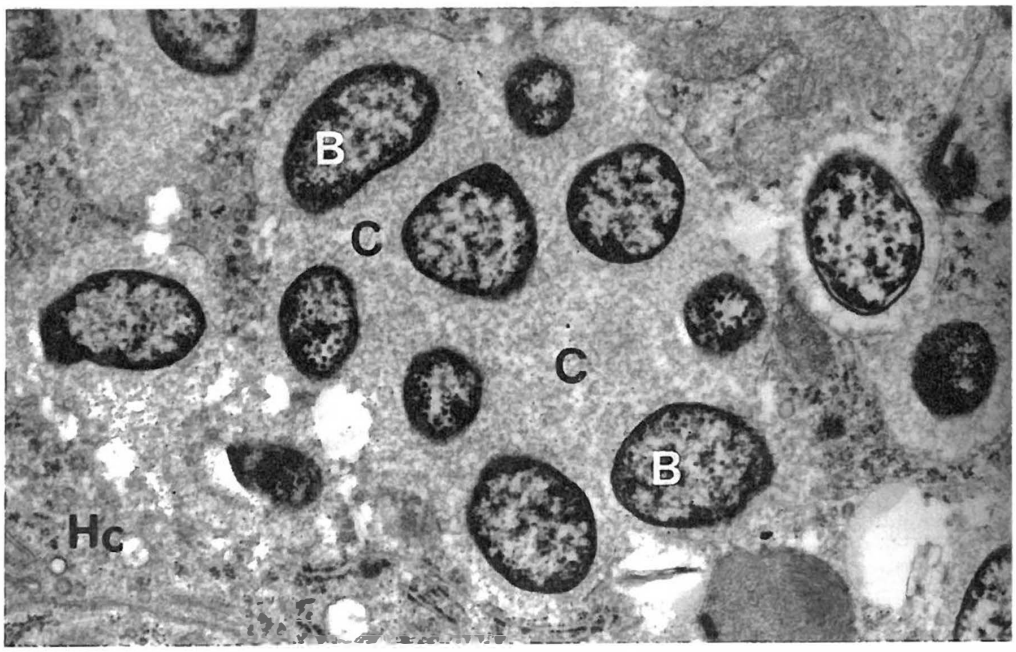

Figure 44: Histiocyte (Hc) containing aggregates of bacilli (B) lying within an amorphous capsular - like material (C). Note individual capsular material is not discernible (Mag x12 000).

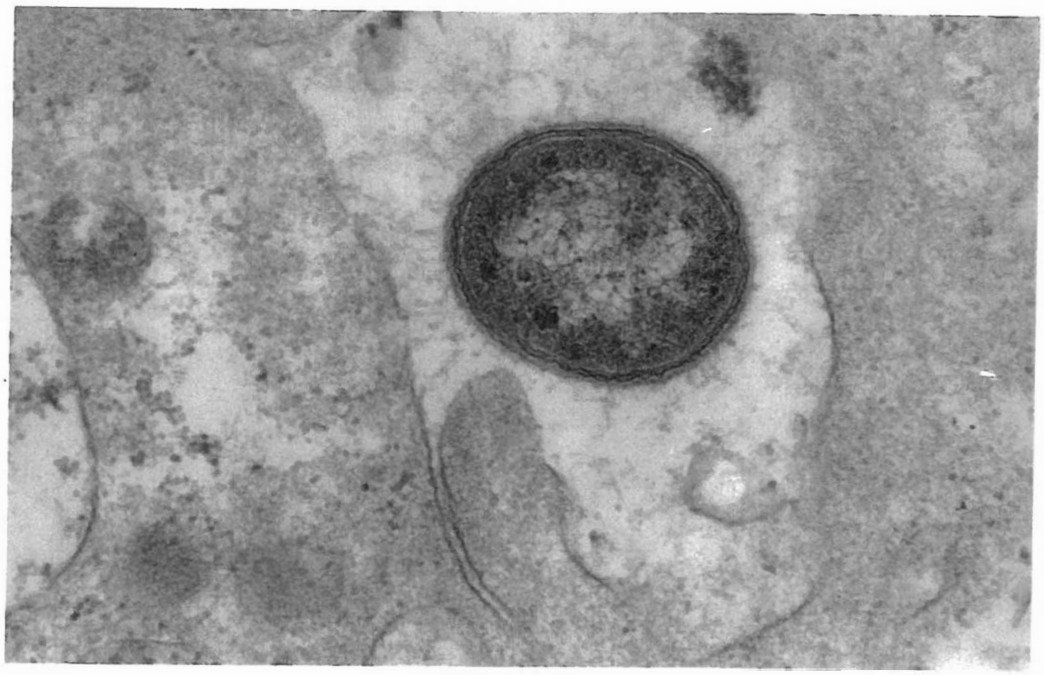

Figure 45: Electron micrograph depicting intercellularly localised bacterium (Mag x30 000).

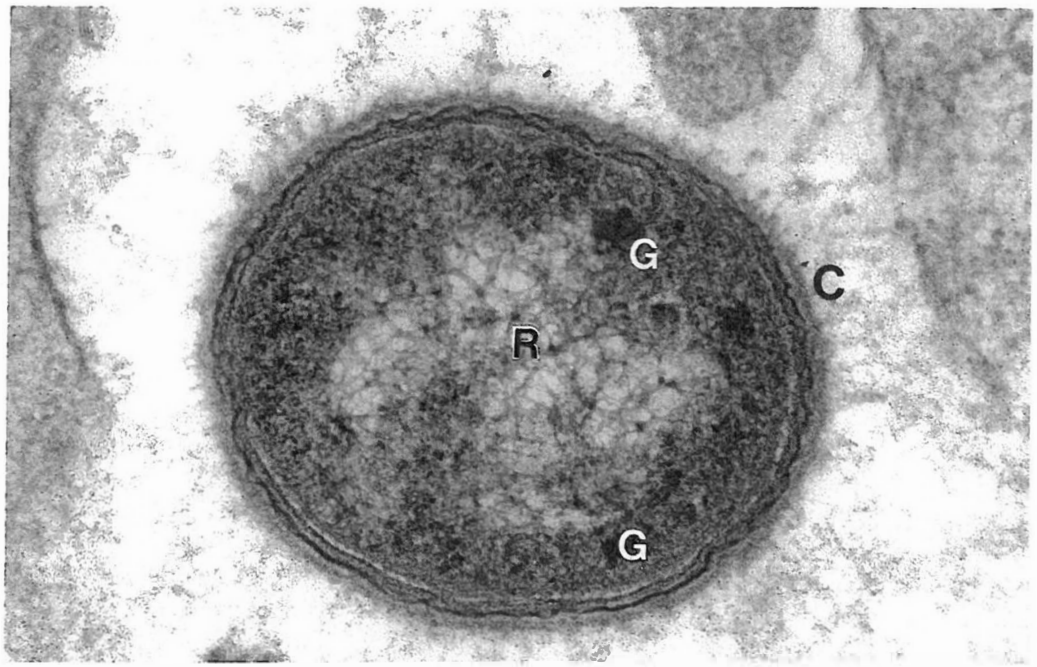

Figure 46: High power magnification of figure 45 showing the filamentous skein of nucleoplasm, prominent electron dense granules (G) and ribosomes (R). Granules are peripherally positioned whilst the nucleoplasm is centrally located. The width of the capsule (C) is markedly decreased (Mag x60 000).

dense granules were prominently visible and located at the periphery of the cytoplasm, whilst the ribosomes were scattered throughout the bacterial body (Figure 46).

The bacterial cell wall consisted of an outer membrane, a middle electron opaque layer and an inner plasma membrane (Figures 47 and 56). The periplasmic space was electron lucent. The outer surface of the bacterial cell wall was corrugated (Figure 46) with a trilaminar structure being clearly visible (Figure 47). The width of the total cell wall was 7nm, with the outer membrane measuring 2nm, the middle electron opaque layer measuring 4nm and the inner plasma membrane measuring 1nm in width (Figure 47).

From the biopsy specimens, the capsule of the bacteria appeared to be well preserved (Figures 46, 48, 49, 50 and 51). The structure of the capsule appeared conspicuous, relatively dense and uniform in thickness around some bacteria (Figures 48 and 50), with the thickness varying between 40nm (Figure 47) to 150nm (Figure 49). The measurements of the capsules were determined from the point where the capsular material was closely adherent to the bacterial body. In some bacteria a faintly contrasted zone of capsular material was evident around a sharp contour of the surface of the outer membrane. This capsular material formed a delicate web-like structure with varying degrees extending from a denser capsule of the surface contour (Figures 46 and 51). These capsules varied in thickness around the bacterial body. The texture of the capsule was fibrous (Figure 50). The capsules of some bacteria appeared to be continuous between adjacent bacteria (Figure 48), whilst a few bacteria showed a firm attachment of the capsular material to the inner surface of the limiting membrane of the phagocytic vacuole by extensions of the capsules as fine fibrillar strands at regular intervals (Figure 48). At a higher magnification the bacteria were clearly surrounded by an electron dense capsule and an electron lucent phagocytic vacuole. The areas of the bacterial body with the trilaminar cell wall structure, the capsule, phagocytic vacuole and the trilaminar limiting membrane were well demarcated as seen in figure 49. All types of capsules were always seen to cover the entire surface of the cell envelope (Figures 46, 47, 48, 50, 51). Occasionally the capsule had separated from the cell surface due to preparation artifacts which showed the bacterial body to be surrounded by a clear area whilst the electron dense area surrounding the clear area had been displaced and pushed to the periphery of the vacuole (Figure 52). The trilaminar

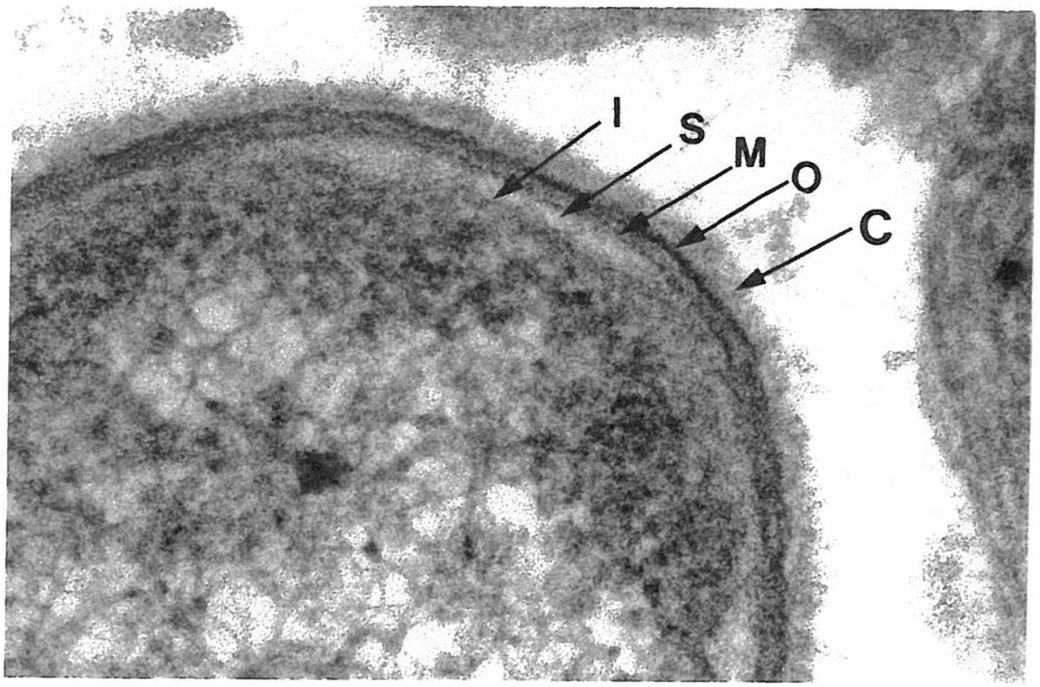

Figure 47: Electron micrograph showing the details of the cell wall structure consisting of an outer membrane (O), middle electron opaque layer (M), inner plasma membrane (I), with periplasmic space (S). Note the narrow electron dense capsule (C) (Mag x120 000).

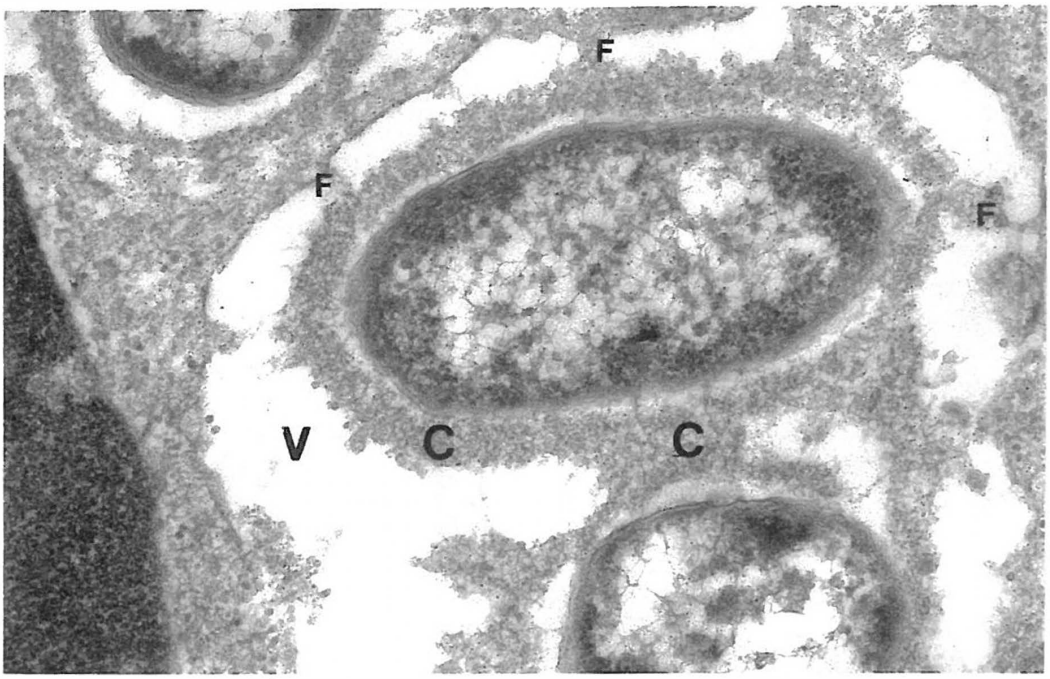

Figure 48: Electron micrograph of a bacilli within a phagocytic vacuole (V). Capsular material (C) between adjacent bacilli seen to be continuous with each other. The fine fibrillar (F) strands are evident at regular intervals attaching the capsule to the limiting membrane of the vacuole (Mag x30 000).

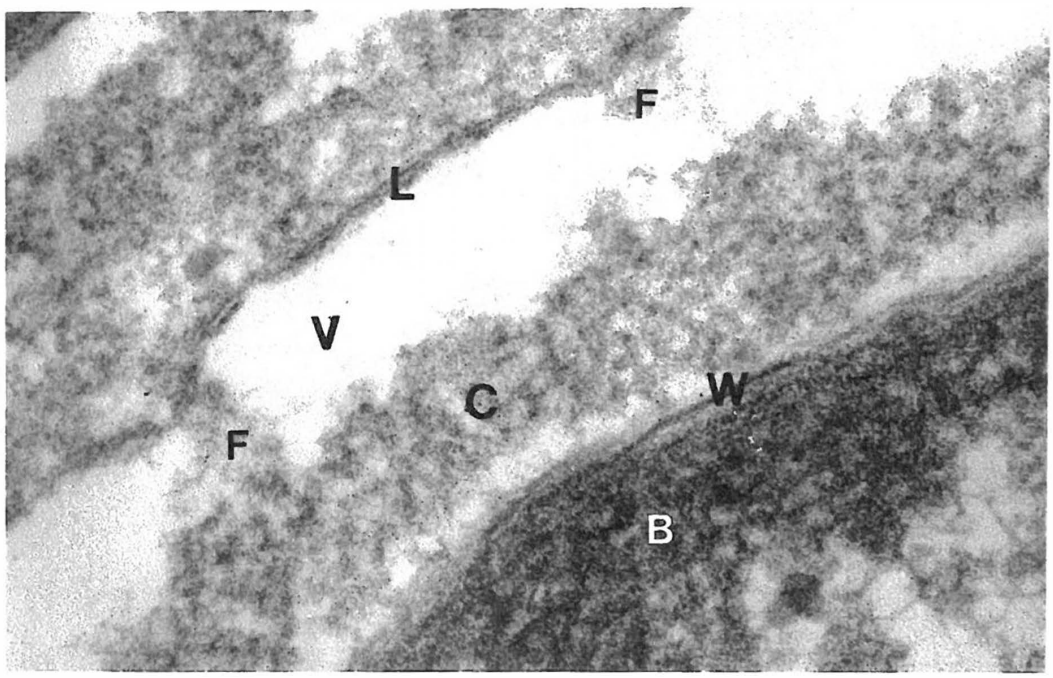

Figure 49: High power electron micrograph of a section of figure 48 showing the limiting membrane (L) of phagocytic vacuole (V). The dense capsule is attached to the membrane by fine fibrillar strands (F). Note the well demarcated bacillus (B), cell wall (W) and capsule (C) (Mag x120 000).

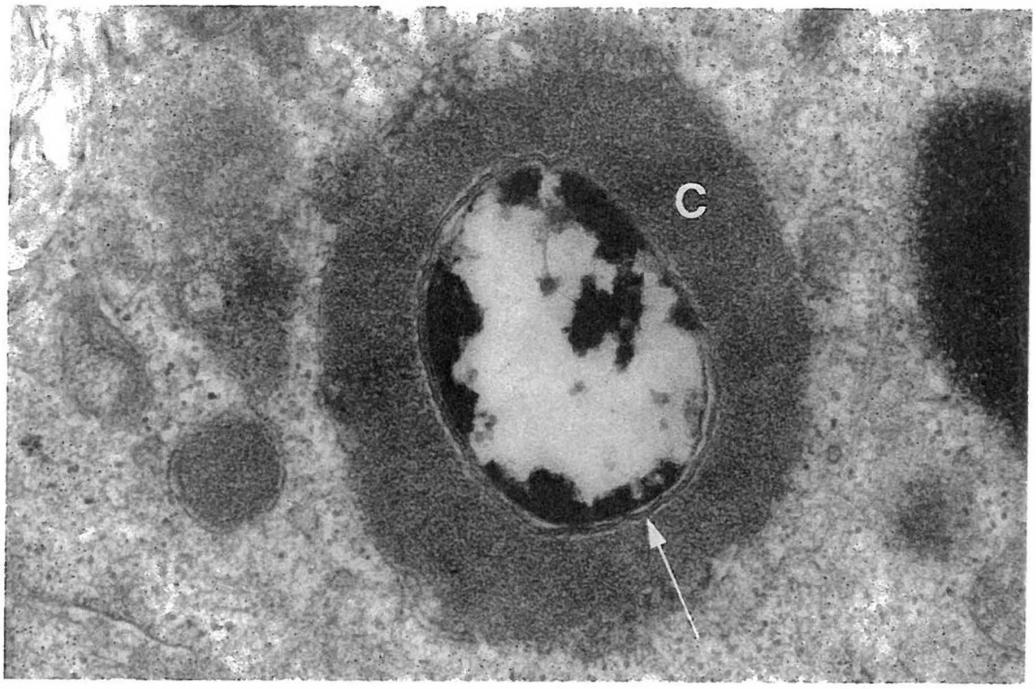

Figure 50: Electron micrograph demonstrating the homogeneous dense capsular material (C) which is adherent to the cell surface. The trilaminar nature of cell wall (arrow) is evident. (Mag x40 000).

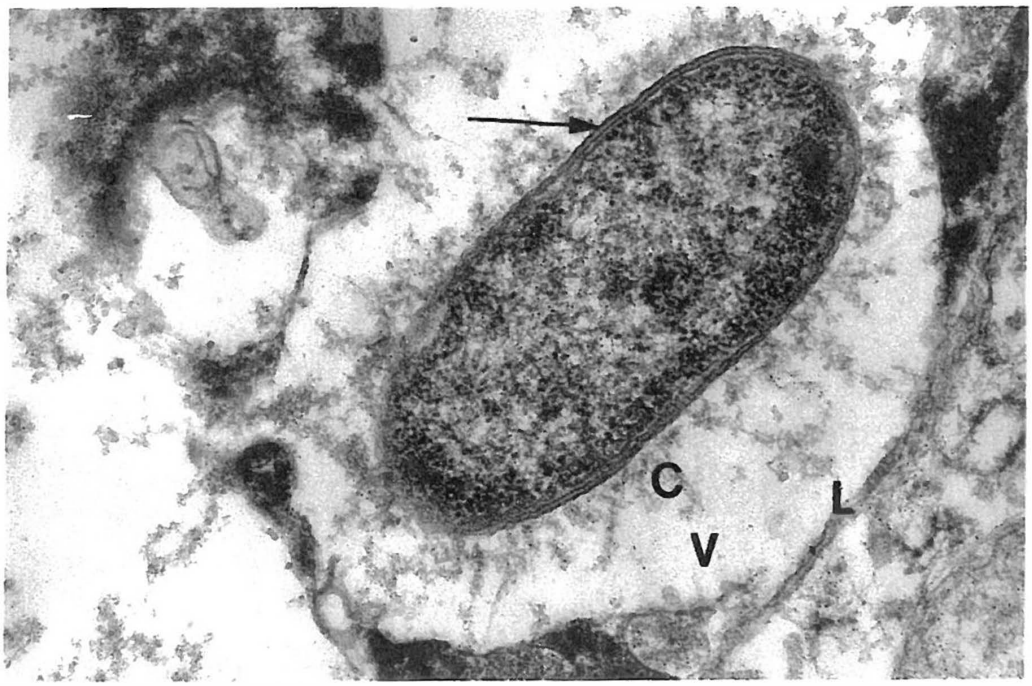

Figure 51: Electron micrograph showing a bacillus within a phagocytic vacuole (V) surrounded by a limiting membrane (L). The trilaminar cell wall structure is evident (arrow). Note the fibrous nature of capsular material (C) (Mag x30 000).

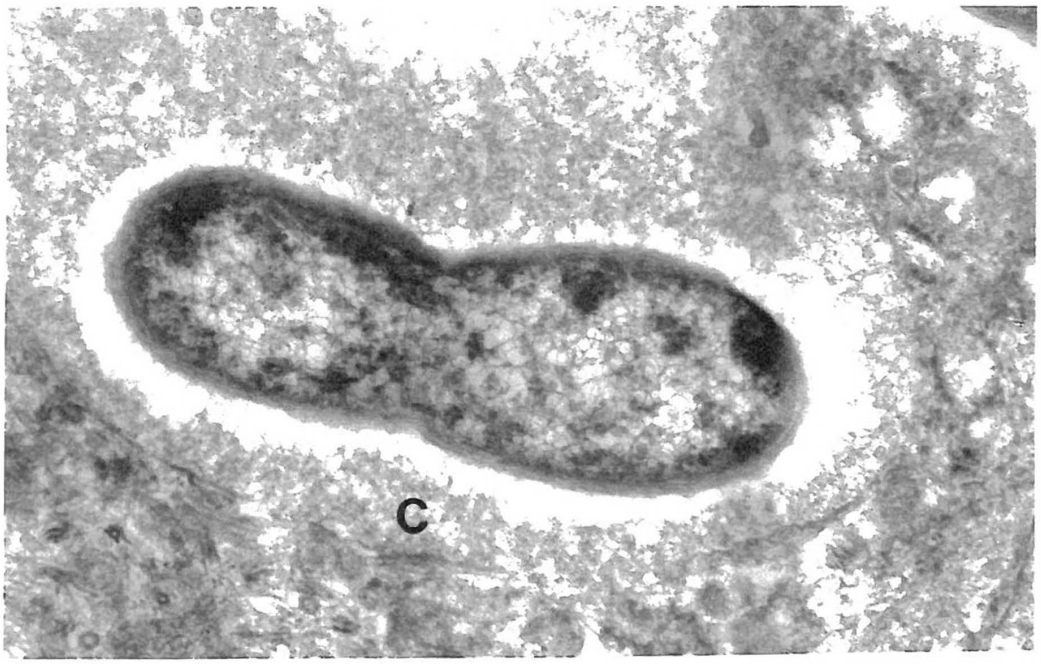

Figure 52: Electron micrograph illustrating a bacterium in the process of division within a phagosome. Note the capsule (C) has separated from the cell surface. The electron dense area (C) surrounding the clear area is being pushed to the periphery of the vacuole (Mag x100 000).

limiting membrane of the vacuole was clearly evident between the capsule and cellular matrix (Figure 52). There was no evidence of intra or extrabacterial particles suggestive of phage like entities or the presence of fimbriae or flagellae.

4.3.2.5 Multiplication of bacteria in tissues

Several bacteria were in the process of cell division. The dividing bacteria were either in an unfused (Figures 53, 54, 55) or fused phagosome (Figure 43). It was clearly visible that the bacteria were seen to divide by the pinching inward of the cell membrane and cell wall, at the point where bacterial septation occurred in later stages (Figure 56). The phagosome within which the bacteria were present, did not divide at the time of bacterial cell division (Figure 43).

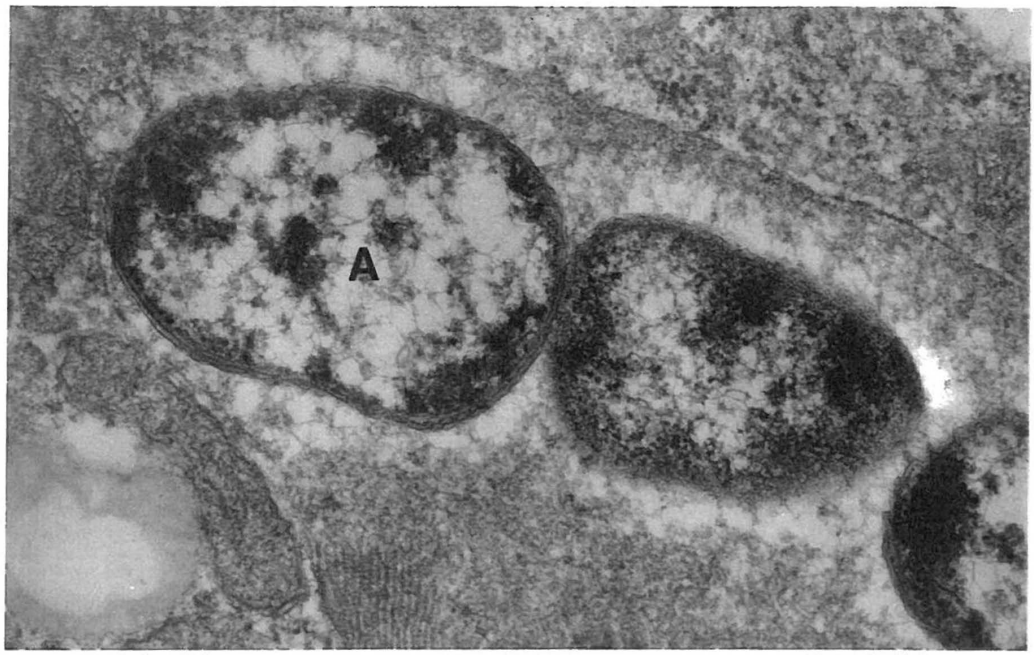

Figure 53: Electron micrograph showing a bacterium within a phagosome in an epidermal cell. Two daughter cells are visible after fission. In bacterium (A) the trilaminar nature of cell wall is evident (Mag x30 000).

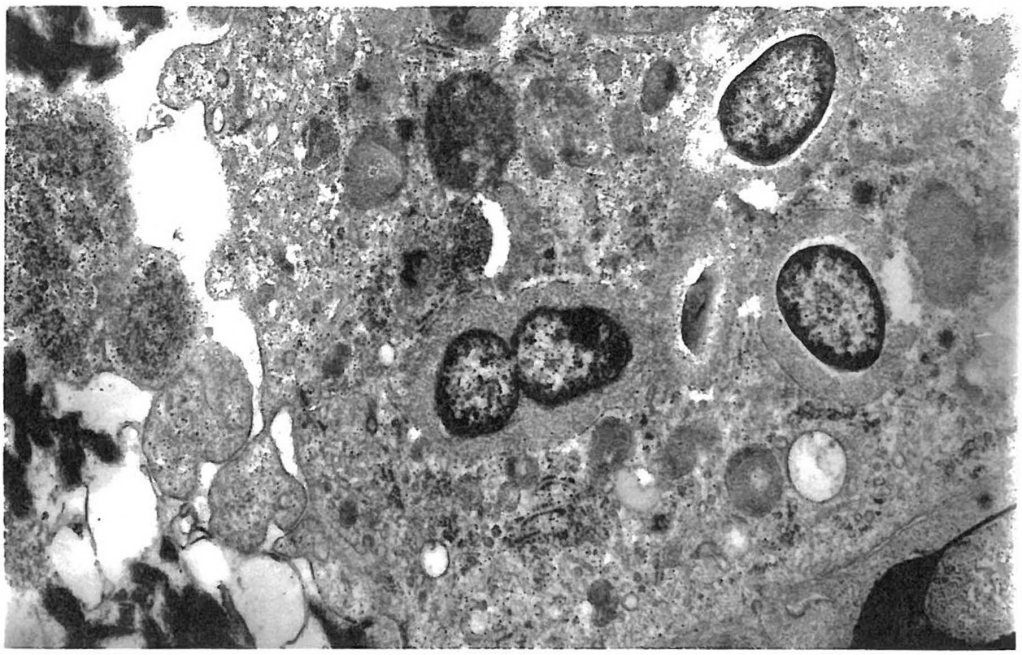

Figure 54: Electron micrograph depicting histiocyte containing bacilli. Individual bacillus capsular material is clearly discernable without the evidence of a phagocytic vacuole (Mag x10 000).

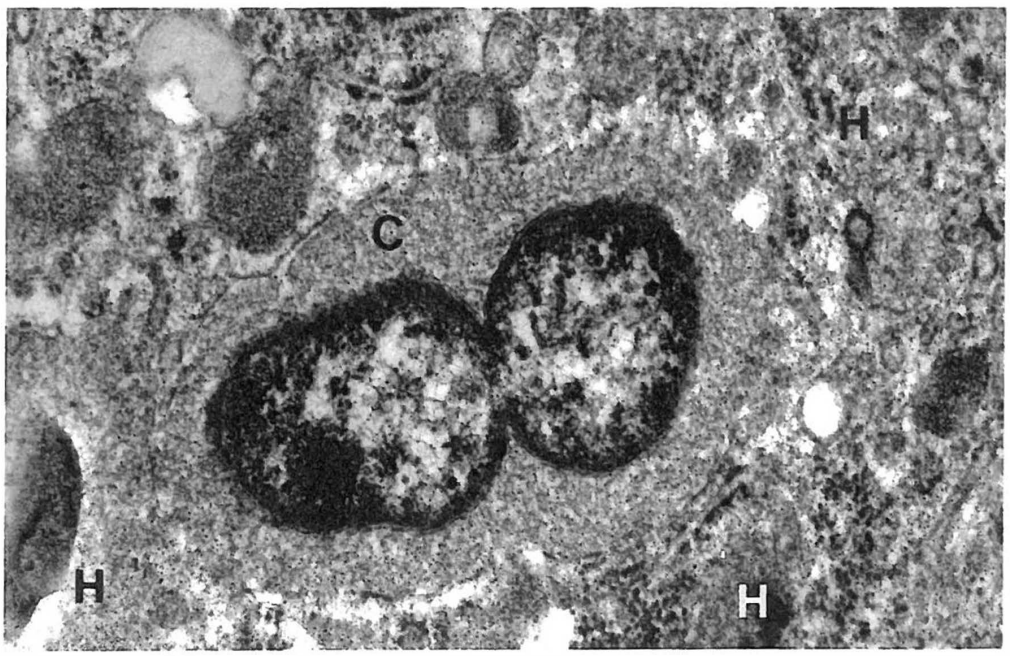

Figure 55: High power electron micrograph of figure 54 illustrating a bacilli undergoing cell division. Capsular material (C) is distinct from the cellular matrix (H) (Mag x25 000).

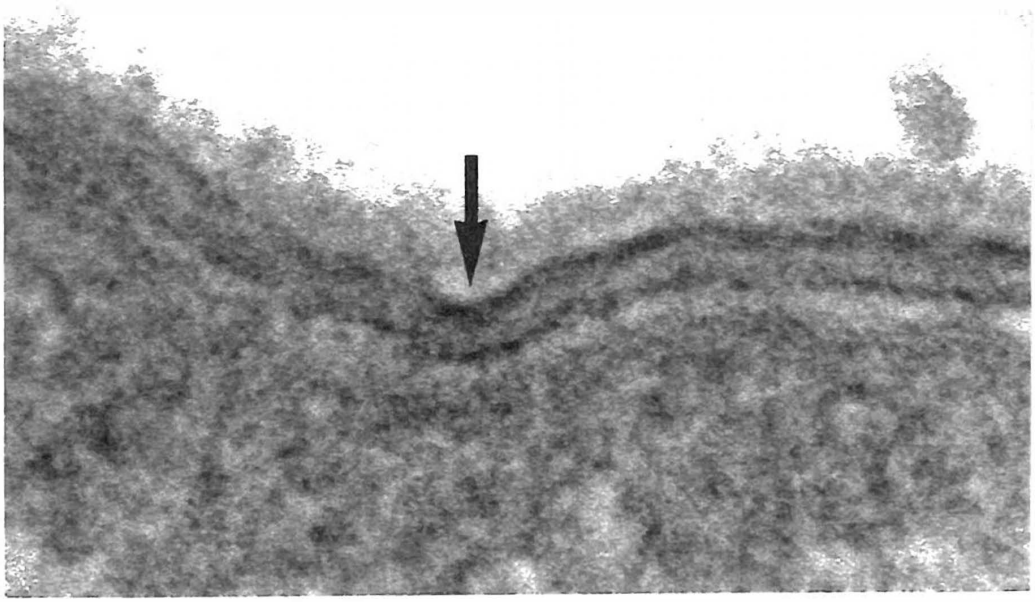

Figure 56: Electron micrograph depicting pinching in of both the cell wall and the cell membrane in a dividing organism. The trilaminar cell wall structure is clearly evident (Mag x200 000).

4.4 DISCUSSION

Previous reports on the description of the ultrastructural features of *C. granulomatis* have been inconsistent and at variance on a number of structures (Davis *et al* 1969; Dodson *et al* 1974; Kuberski *et al* 1980; Chandra *et al*, 1989).

In this study the ultrastructural morphological features of the organism in culture were the same as those seen in tissue biopsy specimens. These being the recognition and positioning of electron dense granules towards the periphery of the cytoplasm, the cell wall structure being typically Gram negative and the capsules being electron dense.

This study demonstrated that the tissue response in granuloma inguinale, is predominantly an accumulation of histiocytes, plasma cells and neutrophils with lymphocytes hardly ever being visualised. The non infected cells showed no evidence of necrosis, whilst infected cells were at various stages of degeneration leading to necrosis. The mechanism by which *C. granulomatis* infects cells, persists within them and induces necrosis is unknown. The culture technique allows the study of such characteristics.

The cytoplasmic inclusions within the bacteria were recognised as distinct electron dense granules and have been reported in previous studies (Davis *et al* 1969; Dodson *et al* 1974; Kuberski *et al* 1980; Chandra *et al*, 1989). These granules are characteristically found in certain species of bacteria and are present as storage material in the form of polymers. Polymers of glucose, in the form of glycogen and starch may be present. Some bacteria store polyhydroxybutyrate in visible granules which usually disappear under restricted nutritional conditions, whilst others produce visible structures of an inorganic phosphate polymer. These granules are prominent in *Corynebacterium diphtheriae* and are known as volutin granules. It could be that in Donovan bodies the electron dense granules observed in the cytoplasm on transmission electron microscopy and the characteristic intense single or bipolar staining appearance with the Giemsa stain on light microscopy are similar storage granules. The nature of these granules for *C. granulomatis* need to be determined as to whether they are similar to the inorganic phosphate polymer of *C. diphtheriae* or contain carbohydrate or lipid polymers.

The ultrastructural appearance of the cell wall in this study was consistent with other reports of the bacterium (Davis *et al*, 1969; Kuberski *et al*, 1980; Dodson *et al*, 1974) and is typical of Gram negative bacteria (Costerton, 1979, Beveridge and Graham 1991).

The presence of the capsule has also been described by several investigators (Davis *et al* 1969; Dodson *et al* 1974; Kuberski *et al* 1980; Chandra *et al*, 1989) who have studied the microbe in host tissue. This description appears to be discrepant in relation to the present study. The capsule has been described to be an electron lucent area around the organism (Davis *et al* 1969; Dodson *et al* 1974; Kuberski *et al* 1980; Chandra *et al*, 1989), whereas the features of the capsule from the monocyte co-cultured specimen were electron dense. Kuberski *et al*, (1980) described the electron lucent area as "presumably a capsule" and "most probably represents capsular material" indicating that there was no clarity in the description of the capsule. It would appear that since previous electron microscopic studies on granuloma inguinale have been performed on clinical biopsy specimens, it is possible that these electron lucent areas were in reality intracellular vacuoles within which the bacteria are situated and not bacterial capsules, or the capsule might have been obscured possibly because of inadequate specimen preservation (Silva and Sousa, 1973; Silva and Macedo, 1982; Spagnola *et al*, 1984) as seen in one of the preparations (Figure 52). The capsules surrounding the bacteria in the biopsy material were very much similar to those observed with the cultured bacteria. Electron lucent areas were also seen, which makes it likely that these areas are indeed artifacts.

The abundant water content of the capsule of many bacteria makes it difficult to preserve the structure of such organelles during dehydration. These are magnified by the propensity of the polysaccharides not to cross-link or stabilise during common fixation procedures. These procedures render the capsule to be rather ill defined with varying degrees of width, contrast and density (Bayer and Thurow, 1977; Beveridge and Graham 1991). In this study the capsules were invariably seen as distinct structures surrounding the bacteria. Occasionally it formed a loose association with the wall, which could represent the capsule in a collapsed state (Bayer and Thurow, 1977). In the absence of a clear visible capsule, the use of electron dense stains such as ruthenium red and alcian blue have been used (Puthucheary *et al,* 1996). These add chemical stability through salt linkage between the

polymers, thereby reducing the degree of collapse during processing (Luft, 1971). The use of specific antisera or lectins, selectively maintains the extended state of the capsule polymers (Beveridge and Graham, 1991). In this study, these stains were not utilised since the capsules were clearly evident, however, the morphology showed varying degrees of the widths of the capsules. The "bleb-like" effect on the capsule of the dividing bacillus from the "cultured" specimens was most probably the typical aspects of shrinkage and distortion of the capsule during sample preparation of fixation and dehydration. The phenomenon has been recognised in *Escherichia coli* (Bayer and Thurow, 1977).

Capsules have a protective function which is important in determining the virulence of an organism (Beveridge and Graham, 1991). It has been suggested by Dodson *et al* (1974) that the variation in the configuration of the capsules may reflect the organisms virulence capacity. Davis *et al* (1969) described the capsule as being unusual, as it is large in size and has a capsular membrane. Whether the membrane like structure observed on the periphery of the capsule in this study represents the capsular membrane described in Davis' study is unclear. The extent of the capsules could also suggest the potentiating effect and the enhancement of the virulence of this organism, thereby rendering its ability to interfere with phagocytosis, thus providing protection against intracellular killing mechanisms and promoting and persisting in an infection within host cells.

Capsule synthesis of bacteria is greatly influenced by growth conditions (Bayer and Thurow, 1977). Using light microscopy De Monbreun and Goodpasture (1938) and Anderson *et al* (1943) reported that the appearance and width of the capsule was enhanced from cultured organisms as compared to those seen in direct specimens. In this study, the appearance of the capsule was also greatly enhanced in "cultured" specimens, whilst the capsule from the biopsied specimens varied considerably in width and density. It is most likely that specific components in the monocyte co-culture system contributed to the enhancement of the capsules. The abundance of capsular material from the cultured specimens could also suggest the increased virulence in association with capsular material since many bacterial capsules degrade to form biofilms which allows groups of bacteria to be embedded within these biofilms, thus allowing the disease to progress (Beveridge and Graham, 1991). There is a clear relationship between capsular type and pneumococcal

disease in humans (Gray and Dillon, 1986) and in pneumococcal infections the capsule is necessary for the virulence and the amount of capsule has an effect on virulence (Watson and Musher, 1990). The ability of pneumococcal isolates to kill mice and the length of time between inoculation and death has been shown to be strongly associated with capsular type (Briles *et al*, 1991). In an *in vivo* study of *Bacillus anthracis* injected into mice, only those organisms known to be virulent had capsules (Roth and Williams, 1964).

The composition of the *C. granulomatis* capsule, whether it is polysaccharide of single or multiple sugars or polypeptide has not been determined. The capsules of most other bacterial organisms such as *Haemophilus influenzae, E coli* are polysaccharide and that of *B. anthracis* and *Yersinia species* are known to be polypeptide. All capsules invariably have the same electron dense character (Costerton, 1979; von Iterson, 1965; Bevridge and Graham, 1991; Puthucheary *et al,* 1996) as observed for *C. granulomatis* from both cultured and biopsy specimens. These observations are in contrast to the reports others for *C. granulomatis* capsules (Davis et a 1969; Dodson *et al* 1974; Kuberski *et al* 1980; Spagnola *et al*, 1984; Chandra *et al*, 1989).

Dodson *et al* (1974) have reported the presence of both capsulated and unencapsulated forms in biopsy specimens. In the present study the ultrastructural characteristics of the capsules from the biopsy specimens clearly showed a marked variation in the morphology. Although an extensive search was made to visualise unencapsulated forms, these were not evident. However, some bacteria displayed capsules, very narrow in width, whilst some displayed extremely fine web like structures. These forms could inadvertently have been recognised and reported as unencapsulated forms.

Davis *et al* (1969) have described the presence of "bacteriophage" attached to the cell wall and empty phage heads within the organism and suggested that *C. granulomatis* is a phage modified species. This phenomenon was confirmed in a subsequent study (Davis, 1970), however, it has been strongly refuted by other workers (Dodson *et al*, 1974; Kuberski *et al* 1980). In the present study there was no evidence of particles either within or on the organism to suggest the presence of bacteriophage. Furthermore there was also no evidence to suggest the presence of fimbriae and flagellae (Chandra *et al*, 1989) or fimbrae

(Chandra and Jain, 1991). However, fibrillar capsular material was observed which seemed to attach the bacteria to the vacuolar membrane. Such fibrils could have easily been confused with fimbriae.

The fact that the phagosome was not seen to be dividing with the bacilli during cell division is in contrast to what has been observed for *Salmonella typhimurium* infected macrophages and similar to *Salmonella cholerae-suis* infected epithelial cells. When *S. typhimurium* replicates within macrophages the bacilli divides together with the pinching inward of the phagosome and division of the phagocytic vacuole (Buchmeier and Hefron, 1991), whilst *S cholerae-suis* replicates within epithelial cells in a large vacuole filled with multiple bacteria (Finlay and Falkow, 1988). These findings suggest major differences in bacterial strategies for intracellular survival within macrophages.

This study confirms the similarity of the ultrastructural features of *C. granulomatis* in cultured and tissue biopsy specimens. The only difference being in the width and density of the capsule. The electron microscopic features of the capsule as observed in this study are in contrast to the reports in the literature, where the capsule has been consistently described as electron lucent. In addition, bacteriophage, fimbriae or flagellae were not observed.

CHAPTER 5.0
MOLECULAR ANALYSIS: AMPLIFICATION AND SEQUENCING OF 16S rRNA GENE OF *CALYMMATOBACTERIUM GRANULOMATIS*

5.1 INTRODUCTION

Conventionally, the basis for the identification of micro-organisms has been their isolation and propagation on laboratory media and confirmation by morphological appearance, biochemical and serological tests. In many situations reliance on these procedures is impractical. Presently, the use of DNA probes and nucleic acid amplification techniques are proving useful for the characterisation of micro-organisms for which culture and serological methods are difficult, expensive or unavailable.

Recently, the rapidly expanding uses of the small subunit ribosomal RNA sequence analysis has offered an opportunity as an alternative approach for phylogenetic, evolutionary relatedness and diagnostic studies (Relman *et al*, 1992a; 1992b). The most commonly used macromolecule to establish organismal phylogenies is the major structural RNA of the ribosomal small subunit (rRNA), which has a sedimentation coefficient of 16S in prokaryote (Schmidt and Relman, 1994). The advantages are that this macromolecule is present in all organisms, it is homologous, highly conserved, not transferred laterally, sufficient in size to permit a statistically significant comparison and accumulates mutations at a slow constant rate, hence are used as "molecular clocks" (Woese, 1987; Olsen and Woese, 1993). This molecule together with the large subunit (23S) rRNA folds in a precise fashion to form ribosomes. These highly conserved structures perform the crucial task of protein synthesis (Gutell *et al*, 1994). The constrained structure and function of ribosomes ensure a reliable clock-like behaviour on the part of rRNA sequences (Woese, 1987; Wilson 1994).

Nucleotide sequence analyses of 16S rRNA or DNA have become extremely useful for the determination of phylogenetic relationships among members of the bacterial kingdom

(Woese, 1987; Relman *et al,* 1992a, Olsen and Woese, 1993). Phylogenies have provided a view of the pathways down which evolution has proceeded and established a logical basis for taxonomic classification. The phylogenetic relationship also aids in the prediction of some properties of otherwise unknown organisms, where certain groups are expected to have properties common to that group. For the observation of most phenotypic characteristics, pure cultures are required and the ability to predict traits is particularly useful in the description of uncultured organisms. Sequence comparison of the 16S rRNA, results in a universal phylogenetic tree with three primary domains of evolutionary descent and are represented as the Archaea (Archaebacteria), Eucarya (Eukaryotes) and Bacteria (Eubacteria) (Woese *et al*, 1990) (Figure 57), the last of which contains all known human prokaryotic pathogens (Woese, 1987; Olsen and Woese, 1993; Relman, 1993) (Figure 58).

Within the 16S rRNA gene the highly variable portions provide unique signatures. The conserved regions of the sequence are found in all bacteria and broad range primers may be designed to recognise these conserved sequences and thus are used to amplify the variable regions (Chen *et al*, 1989; Wilson *et al*, 1990; Weisburg 1991). The use of the polymerase chain reaction (PCR) (Mullis and Faloona, 1987) allows the amplification of 16S rRNA gene sequences from purified bacterial cultures, even in the presence of human DNA which enables the sequence determination and analysis of all products for phylogenetic information of the organism. Database searches with this sequence can help in the identification of the bacteria to species level (Maidak *et al*, 1994). The phylogenetic comparison of rRNA sequences has become a powerful method for the systematic classification of micro-organisms (Fox *et al*, 1980) and even if an organism does not appear in a database, it phylogeny can be determined by the comparison of its sequences with known sequences. The sequences can also be used to design specific PCR primers and oligonucleotide hybridisation probes for diagnostic purposes. The high level of sensitivity afforded by DNA probes and amplification techniques make these techniques the methods of choice for direct detection of microbial DNA in clinical specimens.

This approach has been extended to the identification of "culture resistant" bacterial pathogens (Wilson, 1994) and PCR has been utilised in generating large amounts of 16S rDNA, using broad range bacterial primers which have been designed from 16S rRNA gene

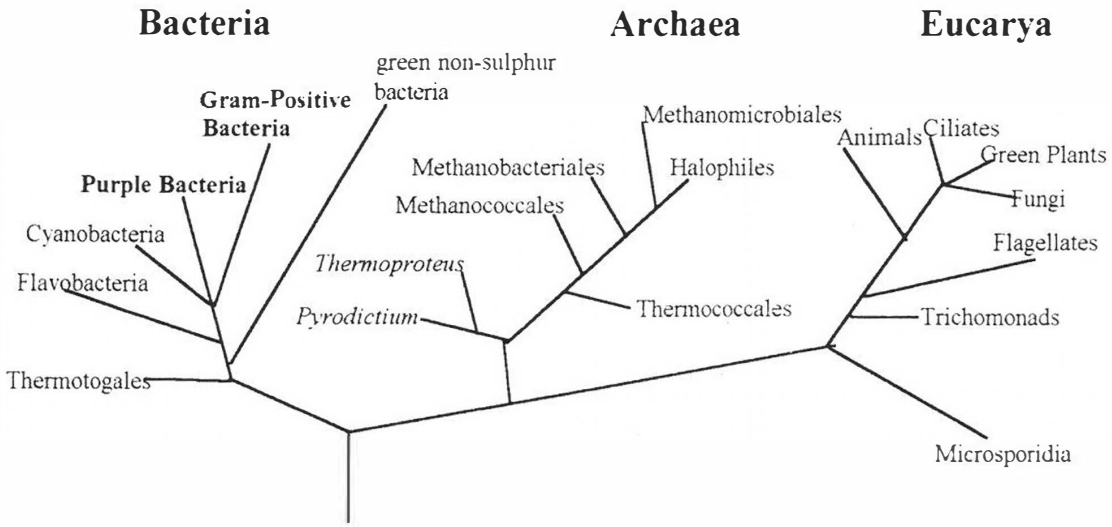

Figure 57: A universal phylogenetic tree based on the comparison of small subunit rRNA sequences, showing the three domains. Line segments reflect evolutionary distance (Woese *et al*, 1990; Olsen and Woese, 1993; Relman, 1993).

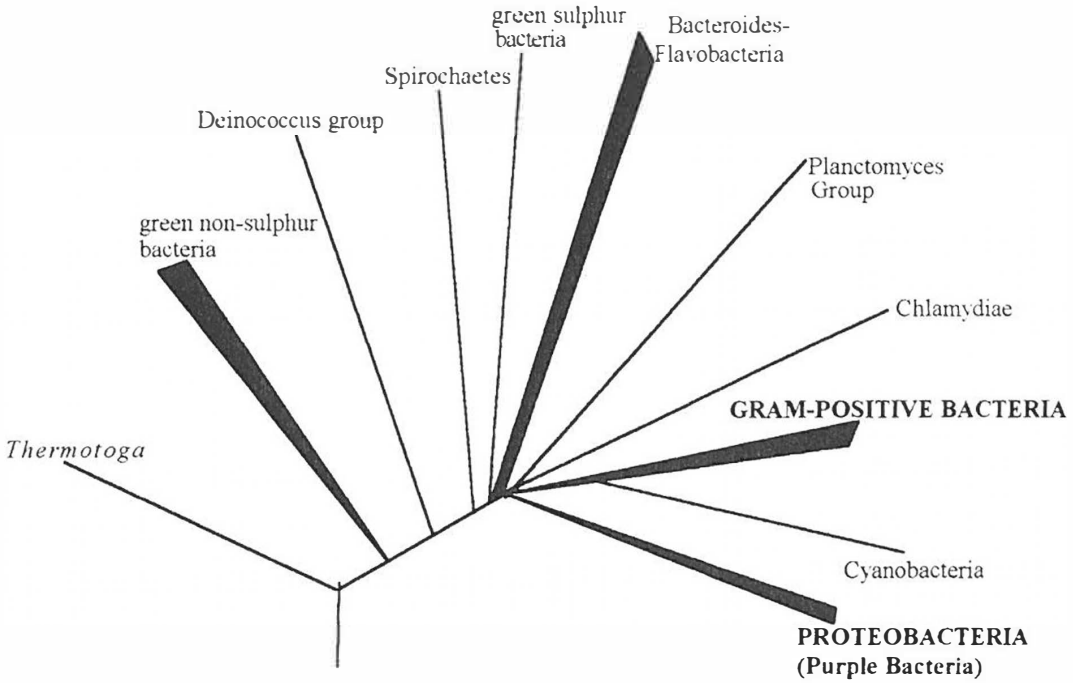

Figure 58: A phylogenetic tree of the domain Bacteria. All currently characterised human prokaryotic pathogens belong to this domain and majority belong to Gram positive or *Proteobacteria* divisions (Woese 1987, Relman 1993).

sequences conserved within the domain Bacteria (Figure 59) (Relman *et al*, 1993). The nucleotide sequences of the primer are complimentary to the target sequences that flank each end. The amplified DNA product contains internal regions of variable sequence that form the basis for specific phylogenetic analysis (Woese, 1987).

PCR was first described in 1983 (Mullis and Faloona, 1987). It is an ingenious *in vitro* procedure based on the ability of DNA polymerase to copy a strand of DNA by elongation of complimentary strands initiated by a pair of closely spaced oligonucleotide primers. The repeated cycles of DNA synthesis results in an exponential amplification of the target nucleic acid.

In the first cycle, the double stranded target DNA sequence is used as template which has specific oligonucleotide primer binding sites. The double stranded target DNA is separated by heat denaturation, and the two primers anneal to their respective recognition sequences in the 5' to 3' orientation. The *Taq* DNA polymerase initiates synthesis at the 3' ends of each primer which results in the extension of the primer via DNA synthesis with new primer binding sites. At the end of the first cycle two copies of the original target DNA molecule are synthesised. In subsequent cycles the original DNA and the newly synthesised DNA strands are used as templates initiating the synthesis of new DNA strands. The products generated accumulate exponentially and the lengths are defined by the distance between and including the primer annealing sites. At present, specific and sensitive detection of micro-organisms can be performed quickly after amplification of DNA by the PCR (Saiki *et al*, 1988). This method allows the detection of a single micro-organism, and it has proven to be of value for diagnosis of infectious agents that are difficult to cultivate (Eisenstein, 1990) and reveal numerous uncultured micro-organisms in natural communities (Ward *et al*, 1990).

Several approaches have been described for the identification of microbial pathogens: direct cloning of mixed population DNA from environmental microbial communities to obtain ribosomal RNA sequences (Ward *et al*, 1990; Schmidt *et al*, 1991); the utility of the PCR in generating large amounts of 16S rDNA from microbial genomic DNA (Edwards *et al*, 1989; Chen *et al*, 1989; Wilson *et al*, 1990; Weisburg *et al*, 1991); and the direct

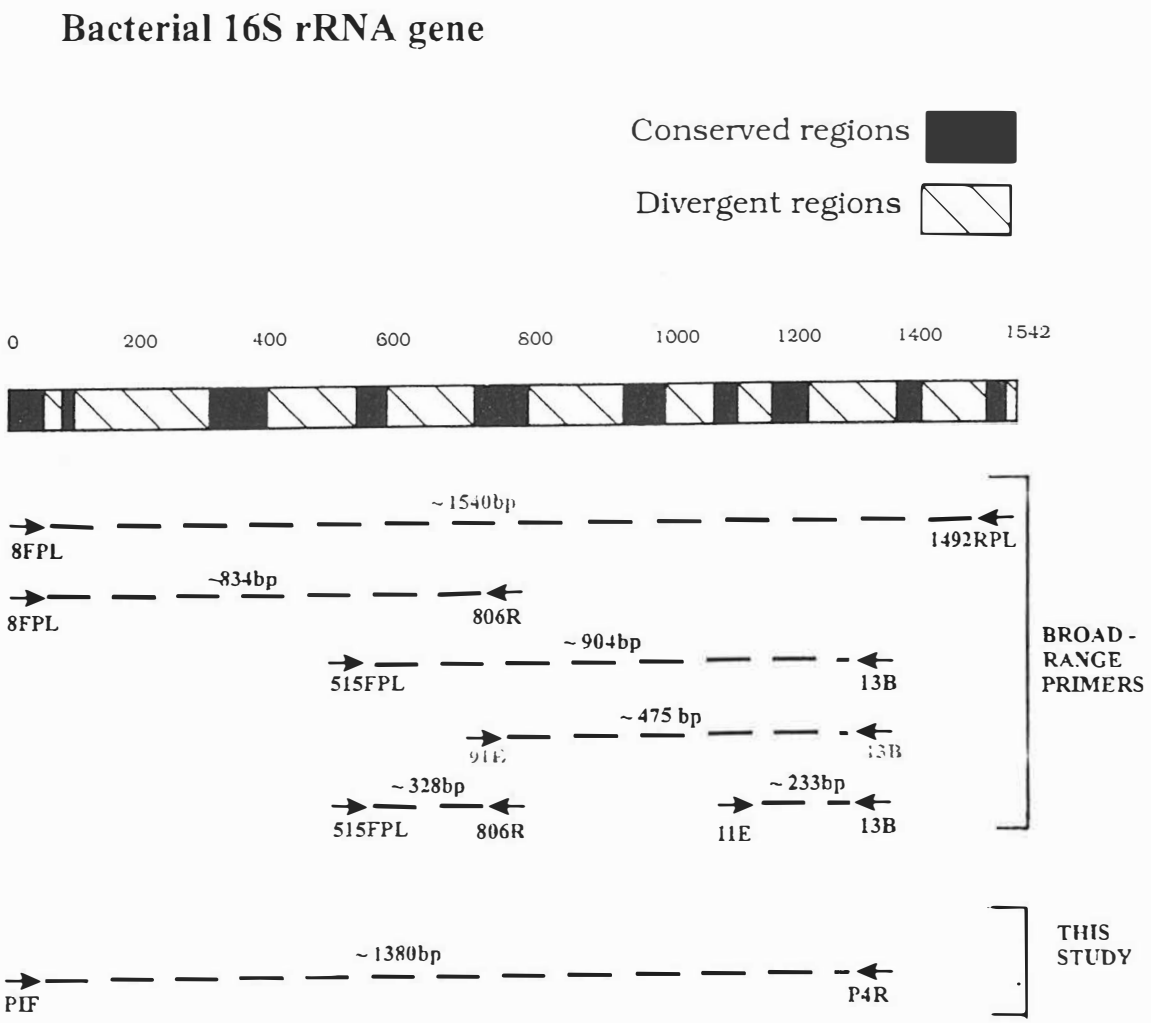

Figure 59: **A schematic diagram of bacterial 16S rRNA gene. Broad range primers are indicated by arrows; dotted lines refer to parts of the gene that are amplified by various pairs of these primers. Broad range primer sequences are highly conserved within domain Bacteria (Relman, 1993). Approximate 16S rRNA nucleotide positions are labelled based on *Escherichia coli* numbering position (Brosius *et al*, 1978).**

amplification of bacterial DNA from infected host tissue (Chen *et al*, 1989; Relman *et al,* 1990; Relman *et al*, 1992b). The procedures for the amplification and sequence analyses of bacterial DNA from tissue specimens are outlined in figure 60.

Nucleic acid amplification techniques are rapid, sensitive and used to identify organisms directly from clinical specimens. The identification of the causative agent of bacillary angiomatosis (Relman *et al*, 1990) and the Whipple's disease associated bacilli (WAB) (Relman *et al*, 1992b) directly from infected tissue has been based on the use of broad range PCR primers in order to amplify a highly conserved region of the gene encoding 16S rRNA and determine the intervening sequence.

The identification of the causative agent of bacillary angiomatosis was based on the observation of Warthin-Starry silver stain positive bacilli, however, four independent patients tissue specimens yielded a 16S rRNA sequence that corresponded to a previously uncharacterised rickettsia-like organism, closely related to *Rochalimaea quintana*, the agent of trench fever (Relman *et al*, 1992b). The diagnosis of Whipple's disease is based on the clinical presentation of the patient with microscopic observation of large macrophages containing diastase-resistant inclusions in periodic acid-Schiff stained infected tissue. These diagnostic procedures are often time consuming and lack sensitivity. However Relman *et al*, (1992b) were able to identify the same organism in five unrelated patients with Whipple's disease and not in 10 patients without the disease. These procedures showed that the WAB was closely related to *Streptomyces* and *Actinomyces* spp. Therefore the amplification and sequencing of 16S rRNA for the identification of "uncultured pathogens" is rapidly expanding.

Calymmatobacterium granulomatis is a fastidious, pleomorphic, Gram negative encapsulated bacillus found intracellularly within macrophages. It is the aetiological agent of granuloma inguinale, a sexually transmitted disease as previously discussed in chapter 2.0. The organism has only been reported to be grown occasionally in embryonated eggs (Anderson, 1943; Anderson *et al,* 1945a) and on cell free media (Dienst *et al*, 1948; Dulaney *et al*, 1948; Goldberg *et al*, 1953; Goldberg, 1962). The methods of these reports have not been reproducible (Maddocks *et al*, 1976) and the fastidious nature of the

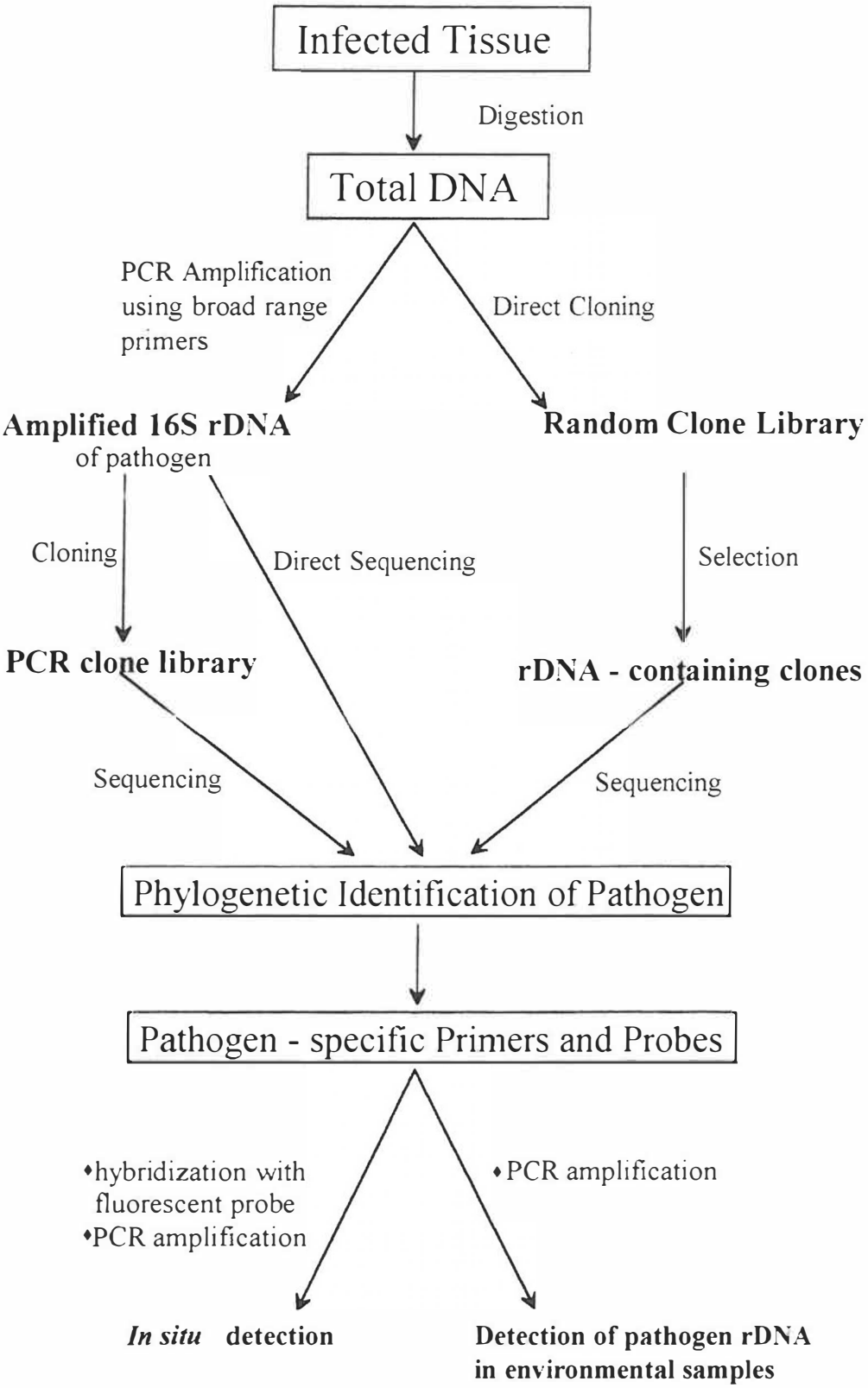

Figure 60: Experimental approach to the identification of uncultured human pathogens using 16S rRNA sequences (Schmidt *et al*, 1991).

organism has resisted its cultivation and thereby hampered its characterisation both phenotypically and genetically.

Recently Bastian and Bowden (1996) using biopsy specimens from the genital lesions of three patients with granuloma inguinale amplified a *Klebsiella* - like sequence targeting the *phoE* gene. This gene encodes a pore forming outer membrane protein (membrane channel) which is highly conserved among members of the family *Enterobacteriaceae.* The DNA sequences encoding these cell surface - exposed regions of outer membrane proteins are genus or species specific (Spierings *et al,* 1992). On the basis of this Bastian and Bowden have suggested the development of probes targeting the *phoE* gene for use *in situ* hybridisation for the diagnosis of granuloma inguinale.

The aims of this study were to:

- amplify bacterial DNA from infected monocyte co-cultures, frozen and paraffin wax embedded tissue biopsy specimens from patients with granuloma inguinale
- clone each of the amplified products in a suitable plasmid vector
- determine the sequence of each of the amplified 16S rDNA
- determine the taxonomic position of *Calymmatobacterium granulomatis*
- hybridise the DNA with published sequences of eubacterial probes

5.2 MATERIALS AND METHODS

5.2.1 SPECIMENS

Tissue biopsy specimens were collected from patients' presenting with ulcerative lesions and in whom a clinical and laboratory diagnosis of granuloma inguinale was made. These were frozen in 10% glycerol peptone broth and / or were formalin fixed paraffin wax embedded. Infected and uninfected monocyte co-cultures (Chapter 3) were used for DNA extraction and amplification. Breast tumour biopsy specimens were used as digestion and negative PCR controls .

5.2.1.1 Frozen tissue biopsy specimens

A piece of frozen tissue was thawed and cut to 3mm x 3mm with a sterile scalpel blade and placed in a 2,0 ml microfuge tube.

5.2.1.2 Formalin fixed paraffin wax embedded tissue biopsy specimen

Using a microtome, several sections of tissue were cut for routine histological examination. Thereafter, a new sterile blade was used to cut 10μm thick sections and placed on alcohol sterilized glass slides. The excess wax was trimmed off and the tissue placed in a 2.0 ml microfuge tube.

5.2.1.3 Monocyte co-culture of *Calymmatobacterium granulomatis*

Monocyte co-cultures (Chapter 3.0) were scraped off the coverslips with sterile Pasteur pipettes and the suspensions centrifuged at 12 000 rpm for fifteen minutes. The deposits were used for DNA extraction.

5.2.2 DNA EXTRACTION

For the extraction of genomic DNA from tissue biopsy specimens a variety of methods are

available. The choice of the method depends on the quality of the DNA required. High quality DNA is generally not required for PCR as is the case for cloning experiments. The DNA isolation method used was that of Relman *et al*, (1990), modified to include the phenol / chloroform extraction procedure, so as to avoid substances which might inhibit the PCR.

5.2.2.1 Extraction of DNA from frozen biopsy specimens

Genomic DNA was extracted from frozen biopsy specimens and breast tumour biopsy specimens and purified by the modification of the method described by Relman *et al* (1990).

The frozen tissue biopsy, and pellets from the formalin fixed paraffin embedded tissue and the deposit from the monocyte co-culture were each resuspended in 600µl of freshly prepared digestion buffer consisting of 500 mM Tris-HCI (pH 9,0); 20 mM EDTA; 10 mM sodium chloride; and 1% sodium dodecyl sulphate with proteinase K (Boehringer Mannheim, Germany) at a final concentration of 1 mg / ml.

The samples were incubated at 56^{0}C for 24 hours. The tubes were briefly centrifuged to collect the condensate and the proteinase K was heat inactivated for 10 minutes at 95^{0}C. The RNA was removed by treatment with Ribonuclease A (Boehringer Mannheim, Germany) at a final concentration of 4,0µg / ml incubated at 37^{0}C for 30 minutes. The crude extract was purified with an equal volume of tris - buffered phenol : chloroform : isoamyl alcohol (25 : 24 : 1 v/v/v) (Sigma, ST Louis, USA), (Saambrook *et al*, 1989).

DNA was precipitated with one tenth volume of 3M Na acetate (final concentration 0,3M) and two and half times volume of ice cold absolute ethanol. The DNA was allowed to precipitate overnight at -20^{0}C. The pellet was washed with 70% ethanol, dried and redissolved in 50 µl of 1x TE buffer (10 mM Tris HCI [pH 8,0]; 1 mM EDTA). DNA concentrations were determined by agarose gel electrophoresis and spectrophotometrically (Genequant Pharmacia Biotechnology, USA). The absorbance of the diluted DNA solution (1:100) was measured at a wavelength of 260 nm and 280 nm relative to TE buffer. The

absorbance at these two readings was used to calculate the ratio which was at least 1,5.

The concentration of DNA was estimated as follows:

A_{260} x 50μg/ml DNA x dilution factor

The final concentration was expressed as μg/ml. It is estimated that an OD of 1 is equivalent to a concentration 50μg/ml of double stranded DNA where this value was used to calculate the DNA concentration in solution.

DNA from the breast tumour biopsy specimen served as digestion and negative controls in the amplification reaction when using broad range bacterial primers.

5.2.2.2 Extraction of DNA from formalin fixed paraffin wax embedded specimen

Genomic DNA isolation from formalin - fixed paraffin wax embedded specimens was performed by the methods described by Wright and Manos (1990) and Kallio *et al,* (1991). Briefly the paraffin wax was extracted twice with 500μl of xylene by mixing gently for 5 minutes at room temperature followed by two washes with absolute ethanol to remove xylene. After removing the ethanol a few drops of acetone were added to each tube. Keeping the tubes open, the acetone was evaporated. For the subsequent steps, the genomic DNA was isolated and extracted as described for frozen biopsy specimens (Section 5.2.2.1).

5.2.2.3 Extraction of DNA from monocyte co-cultures

Genomic DNA from the monocyte co-cultures was extracted using the proteinase K - phenol - chloroform extraction procedure. The deposits of the cultures were suspended in 300μl of digestion buffer and extracted as for frozen biopsy specimens (Section 5.2.2.1). Uninfected monocytes were used as controls for extraction of genomic DNA and as negative controls when using broad range primers for bacterial amplification.

5.2.3 AMPLIFICATION OF 16S rDNA BY PCR

The PCR has advantages in being highly sensitive, fast with a wide range of applications in clinical medicine. However, it has several disadvantages: extreme care needs to be taken to avoid contamination; difficult to design primers for unknown sequences and optimisation of the reaction itself. To improve the specificity and yield of the amplified products, optimal concentrations of $MgCl_2$, *Taq* DNA polymerase, amplification primers, deoxyribonucleoside phosphate and the concentration of genomic DNA in the reaction mixture are required.

5.2.3.1 Preparation for polymerase chain reaction

Three separate areas were designated : clean, grey, and dirty area in strict sequence in this order. Reaction mixtures were prepared in a laminar flow hood which had been irradiated by UV light before and after use in the clean area. A separate set of pipettes and aerosol-guarded tips (Elkay Ultima, USA) were used exclusively for preparation of reactions to avoid contamination. DNA was added to the reaction mixtures in the grey area and amplified in a thermocycler, thereafter the amplified products were detected by electrophoresis in the dirty area.

5.2.3.2 Oligonucleotide primers

The oligonucleotide primers were selected on the basis of published sequences (Relman *et al*, 1990) and were targeted to sites which are highly conserved throughout the eubacterial kingdom but not in eukaryotes and archaebacteria. The primers were synthesized by the solid phase phosphoramidite oligonucleotide synthesis method on a Gene Assembler DNA synthesizer (Pharmacia LKB Biotechnology, USA). The oligonucleotides were purified through NAP 10 columns (Pharmacia LKB Biotechnology, USA) as recommended by the manufacturers. The sequences of the primers are shown in table XI. The forward primer P1F annealed at positions 8 to 27 and the reverse primer P4R annealed at the complement of positions 1390 to 1371 of the *Escherichia coli* numbering system (Brosius *et al*, 1978).

TABLE XI: OLIGONUCLEOTIDE PRIMERS FOR PCR AMPLIFICATION OF BACTERIAL 16S rDNA SEQUENCES

Name	Nucleotide sequence	16S rRNA Positions* 5' to 3'
PIF (Forward)	AGAGTTTGATCCTGGCTCAG	8 - 27
P4R (Reverse)	AGGCCCGGGAACGTATTCAC	1390 - 1371

* *Escherichia coli* 16S rRNA positions which correspond to 5' and 3' ends of each of the primers (Brosius *et al*, 1978).

5.2.3.3 Amplification reaction

DNA extracts from infected monocyte co-cultures and tissue biopsy specimens with *C. granulomatis* were adjusted to a concentration of approximately 400 to 500µg of total DNA / ml. One µl was used in a 100µl reaction volume consisting of 10µl of 10x PCR buffer (100 mM Tris HCl [pH 8,5]; 500 mM KCl); 10µl of 20 mM Mg CI_2; 2µl of 40 mM deoxyribonucleoside phosphate (Boehringer Mannheim, Germany); 1µl (20 pmol) of each amplification primer; 0,5U of *Taq* DNA polymerase (Boehringer Mannheim, Germany) and water to a final volume of 100µl per reaction. Three negative amplification controls were included for each reaction. These consisted of a water control lacking template DNA, genomic DNA from uninfected monocytes and tumour biopsy specimen. DNA extracted from laboratory strains of *Escherichia coli* and *Staphylococcus aureus* were used as positive controls for bacterial amplification.

The reaction mixtures were prepared in thin walled PCR tubes (The Perkin Elmer Corporation, USA) and transferred to an automated DNA thermocycler (Perkin Elmer Cetus 9600; The Perkin Elmer Corperation, USA). The genomic DNA was initially denatured at 95^0C for 4 minutes and amplified over 40 cycles, each cycle consisting of denaturation at 94^0C for 1 minute; annealing at 55^0C for I minute and extension at 72°C for 2 minutes with a final elongation at 72^0C for 7 minutes. All tubes were held at 4°C until further analysis.

5.2.3.4 Detection of PCR products

DNA fragments were resolved using agarose gel electrophoresis according to molecular weights. Smaller fragments migrate faster than larger ones, and the distance migrated on the gel varies inversely with the logarithm of the molecular weight. The size of the fragments were determined by using known size standards and comparing the distance, the unknown fragment had migrated.

Ten microlitres of PCR products were mixed with 2,0µl of 5x gel loading buffer, loaded into the wells of a submerged 1,5% NuSieve agarose gel made up with 1x Tris - borate

(TBE) buffer containing ethidium bromide (final concentration 0,5μg/ml) and electrophoresed using the Hoefer horizontal apparatus (Hoefer Scientific Instruments, San Francisco, USA) at $10Vcm^{-1}$ in 1x TBE running buffer. The bromophenol blue dye front was electrophoresed to the bottom of the gel. The gel was examined by ultraviolet illumination for the presence of DNA bands and photographed on a UV transilluminator using a Polaroid camera and documented on Image store 5000 Gel documentation system (Ultraviolet Products, Ltd).

5.2.3.5 Purification of PCR products for cloning

The desired 1380 bp PCR products were excised using a sterile scalpel blade in the smallest volume of agarose. The gel slices were placed in microfuge tubes and purified using the Qiagen kit (Qiagen Inc. USA). Three hundred microlitres of QX1 (solubilisation buffer) were added per 100mg of gel to solubilise the gel. The Qiaex suspension consisting of silica gel particles were vortexed to obtain a homogeneous suspension. Ten microlitres of the Qiaex suspension was added to the DNA mixture vortexed and incubated at 50^0C for 10 minutes. The samples were vortexed intermittently to keep the Qiaex in suspension and to maximise binding efficiency. Upon solubilisation the DNA was liberated from the agarose and bound to the Qiaex particles in suspension. The tubes were centrifuged for 30 seconds at 15 000 rpm and the supernatant removed and discarded.

The Qiaex pellet containing the DNA was washed twice with 500μl of QX2 (high salt buffer) and centrifuged for 30 seconds at 15 000 rpm. The supernatant was discarded to remove any residual agarose contamination. The pellet was washed twice by resuspending in 500μl of QX3 (wash buffer) and centrifuged for 30 seconds at 15 000 rpm. The ethanol in the QX3 removed all of the salt contaminants.

The pellet was thoroughly air dried to ensure proper elution and resuspended in 10 μl of TE buffer, vortexed and allowed to stand at room temperature for 10 minutes, flicking the tubes every 2 minutes. The eluted DNA was recovered by centrifugation at 15 000 rpm for 30 seconds. The supernatant containing the DNA was transferred to a clean tube. The elution step was repeated with an additional 10μl of TE buffer and the supernatant were

pooled and stored at -20^{0}C and used when required. The purified 1380bp DNA fragment was electrophoresed in a 1,5% NuSieve agarose gel made up with 1x TBE, containing ethidium bromide, to confirm its purity and concentration.

5.2.4 CLONING OF PCR PRODUCTS

Generally, PCR can be used to produce a large amount of specific DNA, however cloning of these PCR products has not been straight forward. Restriction endonuclease sites are often incorporated into the oligonucleotide primers used for amplification, so that cleavage of the product will create sticky ends that can be ligated to an equivalently cut vector. Blunt end cloning often fails due to existence of an unpaired adenosine residue at the 3' end of the fragment, which is added by the *Taq* polymerase to a proportion of amplified molecules. The cloning of blunt ended fragments requires the enzymatic removal of 3' overhangs.

A rapid system for the direct cloning of PCR products has been described by Marchuk et al (1992). This system exploits the template - independent activity of thermostable polymerases *(Taq* polymerase) which preferentially adds a single adenosine nucleotide overhang to the 3' end of the PCR products during PCR reaction (Clark, 1988). Such products can then be inserted directly into a specially prepared compatible vector containing a single thymidine at the 3' end of each fragment (T - vector). The vector and PCR product have complementary single based 3' thymidine overhangs. Self ligation of the vector is prevented by the 3' thymidine overhang. Concatamerization of the insert is prevented by the unphosphorylated 5' end contributed by the oligonulceotide primer and the 3' adenosine overhang added by the *Taq* polymerase during PCR reaction.

The purified amplicons were cloned using pMOS*blue* T - vector kit. (RPN 1719, Amersham Life Science, UK). This kit provides an efficient system for direct cloning of PCR products. pMOS*blue* T - vector has been specifically constructed for this application and has been prepared for T - cloning by digestion with *Eco*RV followed by the addition of 3' thymidine residue which readily ligates with the amplified DNA. The cloning strategy and structural map of the pMOS*blue* T - vector are shown in figures 61 and 62 respectively.

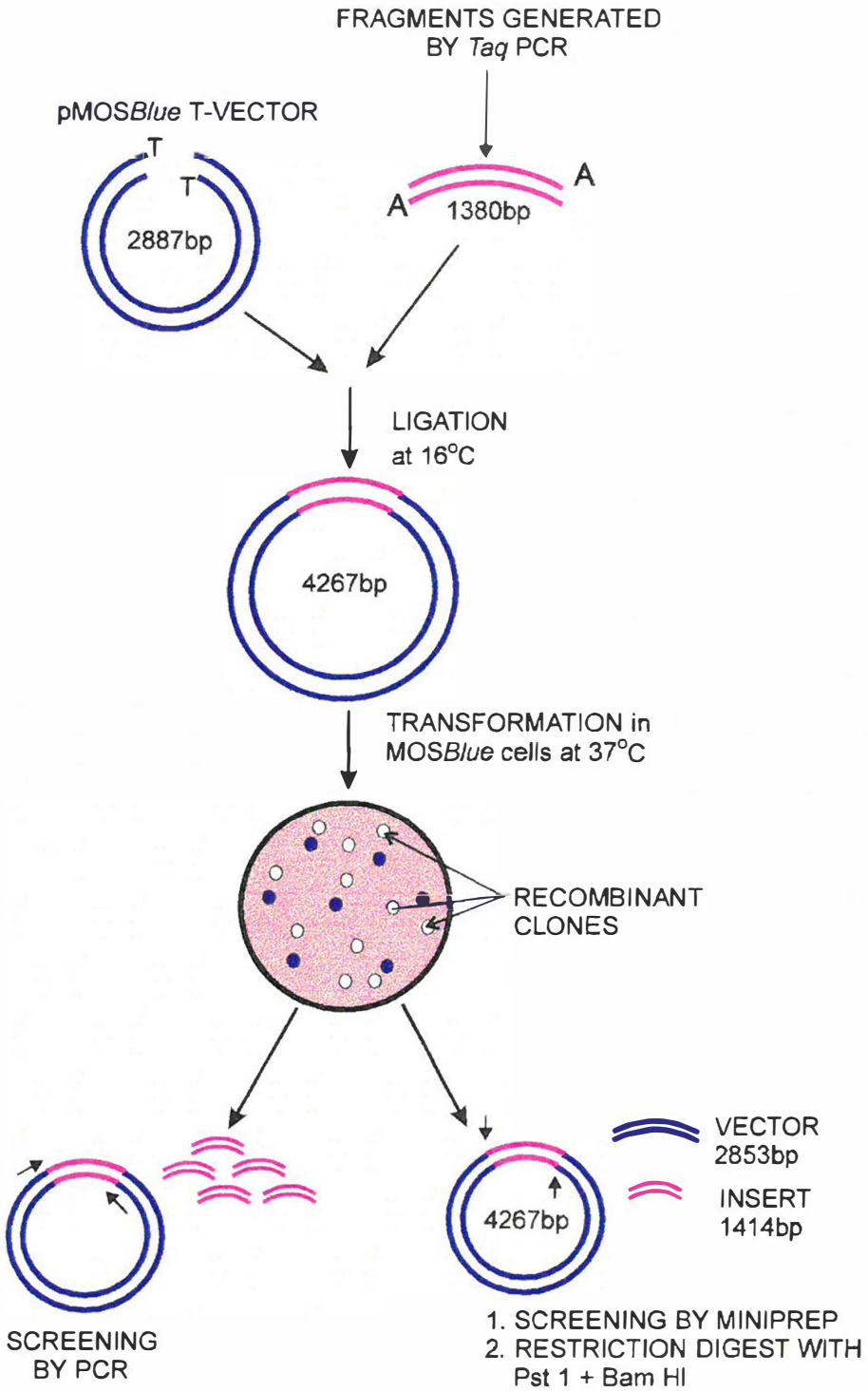

Figure 61: Diagrammatic representation of the T:A cloning methodology

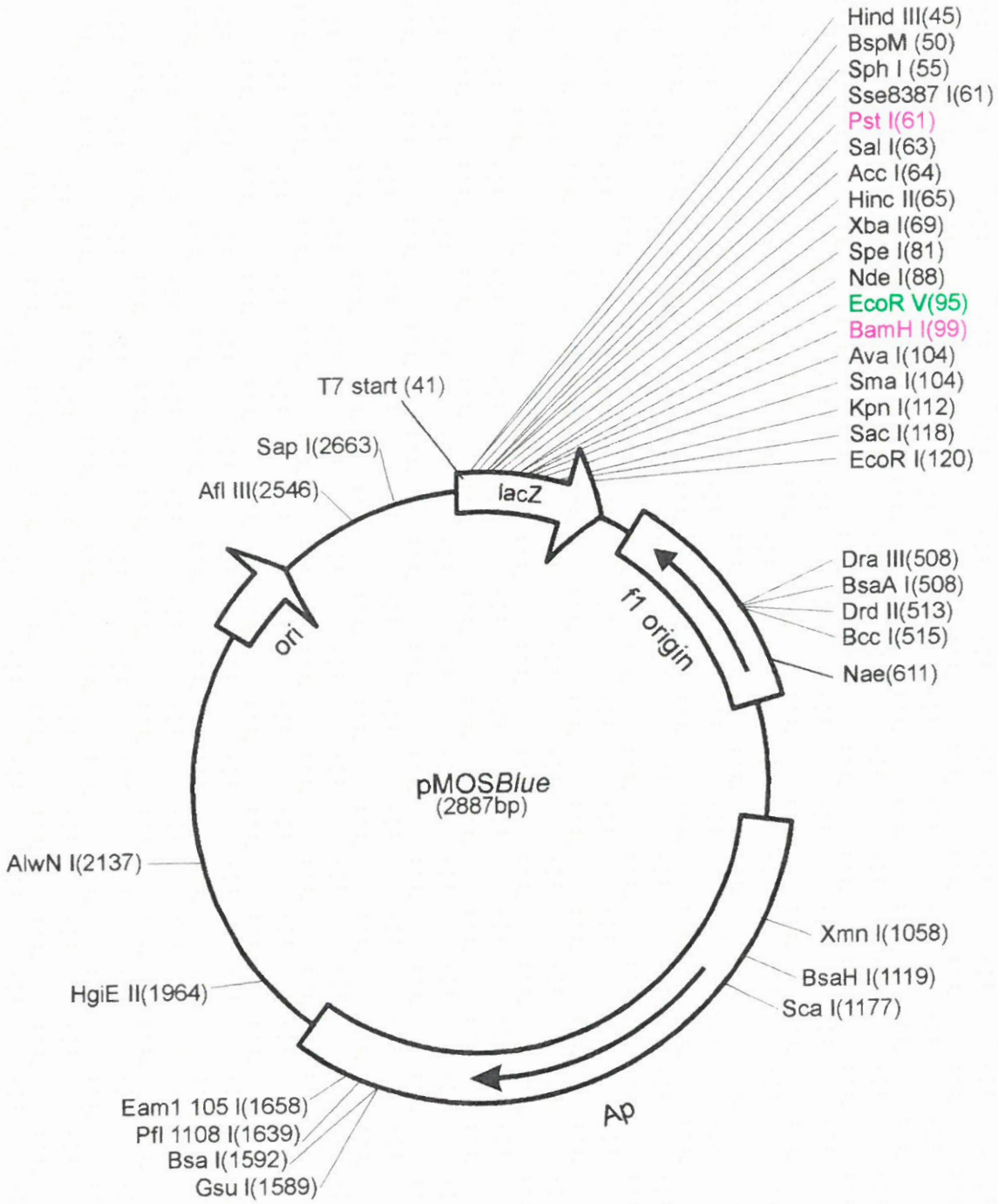

Figure 62: Structural map of pMOS*blue* T - vector.

5.2.4.1 Ligation of PCR products into pMOS*blue* T - vector

The concentration of the purified PCR amplicons were estimated using the Genequant and also compared to the intensity of illumination of DNA standards on an ethidium bromide stained agarose gel. For optimal cloning efficiency the recommended vector to insert ratio was 1 : 7 and therefore 178 ng of the purified insert was required to obtain a ratio of 1 : 7. All ligation reactions were set up in triplicate using vector to insert ratios of 1 : 5; 1 : 7; and 1 : 10. For each PCR product the ligation reactions were set up as follows.

Volume	**Reagents**
1 µl	10 x ligase buffer
0,5 µl	100 mM dithiothreitol (DTT)
0,5 µl	10 mM Adenosine triphosphate (ATP)
1,0 µl	50 ng/µl vector
"X" µl	amplified product (239, 167 and 119 ng/µl)
0,5 µl	T_4 DNA ligase (2 - 3 Weiss units)

Made up to 10µl with nuclease free water.
The reactions were gently mixed with a pipette tip and incubated at 16°C overnight. A positive ligation control consisting of a known insert provided in the pMOS*blue* T - vector kit was included with each batch.

5.2.4.2 Transformation of ligated samples into competent cells

This procedure transforms bacterial cells with circular DNA plasmid containing an origin of autonomous DNA. The growing cells are made permeable and the DNA is taken up from the surrounding buffer. The uptake is facilitated by a brief heat shock. Bacterial cells that have taken up the plasmid DNA are then selected for growth on Lauria Bertonni (LB) agar medium containing ampicillin and tetracycline with X - gal (5-bromo-4-chloro-3-indolyl-B-galactoside) (Boehringer Mannheim, Germany) and IPTG (isopropyl B-D-thiogalactopyranoside) (Boehringer Mannheim, Germany). Cells not containing the plasmid will be detected as blue colonies as the *lac Z* gene has not been interrupted and the IPTG

can convert X - gal to blue colour, whereas successful transformants will form discrete white to colourless bacterial colonies where the *lac Z* gene has been interrupted. .

One µl of the ligated samples were transformed into 20µl pMOS*blue* competent cells. The mixture was gently stirred and allowed to stand on ice for 30 minutes. To test the efficiency of the MOS*blue* competent cells, 0,2ng of test plasmid was added in place of ligation mix. The cells were heat shocked for exactly 40 seconds in a 42^{0}C waterbath and placed on ice for 2 minutes, 80µl of SOC medium was added to each tube and shaken at 250 rpm at 37^{0}C for 1 hour. All reactions were screened for blue / white recombinants by spreading out 50µl of each transformation mixture onto LB agar plates containing 50 µg/ml ampicillin (Smith, Kline and Beechams Pharmaceuticals, UK) and 15µg/ml tetracycline (Upjohn Company, USA) which had been prepared by spreading out 35µl of X - gal (50 mg/ml in dimethylformamide) and 20µl of IPTG (100 mM) per plate. Plates were incubated at 37^{0}C for 18 hours.

5.2.4.3 Screening of recombinant clones

X-gal and IPTG indicator plates containing ampicillin and tetracycline were examined for recombinant colonies which appeared colourless to white. Several recombinant colonies (colourless) and a few non - recombinant colonies (blue) were used to extract and isolate plasmid DNA by the standard alkaline lysis method of Birnboim and Doly (1979).

5.2.4.3.1 *Confirmation of cloned inserts by rapid alkaline lysis plasmid extraction method*

Three millilitre of LB broth containing ampicillin (final concentration - 50µg/ml) (Smith, Kline and Beechams Pharmaceuticals, UK) was inoculated with recombinant colony. The tubes were shaken at 37^{0}C for seven hours, thereafter 1,5 ml was transferred to an Eppendorf tube and centrifuged for 15 min at 12000 rpm. The supernatant was aspirated leaving a dry pellet. The pellet was resuspended in 100µl of ice cold solution 1 (25mM Tris pH 8,0; 10mM EDTA pH 8,0; 50mM glucose). The pellet was vortexed to resuspend the cells. Two hundred microlitres of freshly prepared solution 2 (0,2N NaOH; 1% SDS)

was added to lyse the cells. The tubes were rapidly inverted several times and stored on ice. One hundred fifty microliter of ice cold solution 3 (5M K acetate; glacial acetic acid) was added. The tubes were vortexed, inverted several times and stored on ice for 5 minutes. The plasmid DNA was extracted with 400µl of Tris buffered phenol : chloroform : isoamyl alcohol (25 : 24 : 1) (Sigma, ST Louis, USA). The aqueous phase was carefully removed taking care not to disturb the interphase. The plasmid DNA was precipitated with 800µl of absolute ethanol at room temperature, allowed to settle for 5 minutes and spun for 10 minutes at 12 000 rpm. The pellet was washed with 70% ethanol and spun for 5 minutes. The supernatant was aspirated and the pellet air dried for 20 minutes, thereafter dissolved in 25µl of 1x TE buffer containing Ribonuclease A (20µg/ml) (Boehringer Mannheim, Germany).

DNA preparations from several recombinant clones showed the presence of the insert as upon gel analysis a high molecular weight fragment (4267 bp) was seen as compared to non - recombinant clones (2887 bp).

5.2.4.3.2 *Restriction analysis of recombinant clones*

To confirm with accuracy the presence of the inserts, recombinant clones were digested with restriction enzymes *Bam*HI (restriction site 5' **G▼GATCC** 3'; figure 62) (Boehringer Mannheim, Germany) and *Pst*I (restriction site 5' **CTGCA▼G** 3'; figure 62) (Boehringer Mannheim, Germany). Restriction with these two enzymes should yield a 2853bp fragment (vector) and a 1414bp fragment (insert - 1380bp + partial vector sequence - 34bp). Approximately 5µg of recombinant plasmid DNA was mixed with 1 µl of 10x restriction enzyme buffer, and a combination of 1µl of *Bam*HI (5 U/l) and 1 µl *Pst*I (5 U/l) was added and made up to a total volume 10µl with sterile water. The contents were mixed, briefly centrifuged at 12 000 rpm and incubated at 37^0C for 1 hour. The reaction was stopped by placing on ice. The restriction fragments were analysed by electrophoresis on 1.5% NuSieve agarose gel in 1x TBE running buffer containing ethidium bromide (0,5µg/ml) and photographed using UV transillumination. DNA sequencing of recombinant clones containing the required insert was undertaken.

5.2.5 HYBRIDISATION AND DETECTION

Presently, DNA probes are most useful for the characterisation of micro-organisms for which culture and serologic methods are difficult, expensive or unavailable. When single stranded DNA is placed in contact with a complimentary single stranded DNA sequence, the two DNA molecules bind to each other by hydrogen bonding between the two bases. This binding or hybridisation, forms the basis of the technique used for the detection and confirmation of specific nucleic acid sequences. After southern blotting the restricted cloned fragments were subsequently hybridised separately to published sequences of the universal bacterial, Gram negative and Gram positive probes in order to confirm the nucleotide sequence obtained. For the hybridisation and detection tests, the DIG Oligonucleotide 3' - end labelling kit and the DIG nucleic acid detection kits were used (Boehringer Mannheim, Germany).

5.2.5.1 Oligonucleotide probes

The oligonucleotide probes used to confirm the sequences were available as published sequences and shown in table XII (Griesen *et al*, 1994).

5.2.5.2 Labelling of oligonucleotide probes

The oligonucleotide probes were labelled using the 3'- labelling method, which is based on the addition of digoxigenin (DIG) dideoxyuridine triphosphate (ddUTP) to the 3' end of the oligonucleotide DNA template (probe) with the aid of the enzyme terminal transferase (Boehringer Mannheim) for 15 minutes at 37^0C. The DNA molecular weight marker used for all hybridisation experiments was DIG labelled.

TABLE XII: NUCLEOTIDE SEQUENCES AND LOCATIONS OF PROBES USED FOR HYBRIDISATION

Probe	**Nucleotide sequence**	**16SrRNA Positions* 5' to 3'**
Universal bacterial probe RDR245#	GTACAAGGCCGGGAACGTATTCACCG	1369 - 1395
Gram negative probe DLO4#	GACGTAAGGGCCATGATGACTTGACGTC	1190 - 1217
Gram positive universal probe, RWO3#	GACGTCAAATCATCAGCCCCTTATGTC	1190 - 1217
CR289$ (sense strand) *C.granulomatis* specific probe	TAGTAGGTGGGGTAACGGCT	238 - 257
CF308$ (antisense strand) *C.granulomatis* specific probe	CCGAACCGCTGGCAACAA	1100 - 1117

* Nucleotide numbering based on *Escherichia coli* 16S rRNA positions which correspond to 5' and 3' ends of each probe (Brosius *et al*, 1978).

\# Griesen *et al*, 1994

$ this study

5.2.5.3 Transfer of DNA to nylon membrane

The DNA from the agarose gel was transferred to the nylon membrane by vacuum, because of its efficiency and the short period of time required for complete transfer. After electrophoresis the gel was immersed in denaturation solution (0,5 N NaOH; 1,5 mol/L NaCl) twice for 15 minutes each to denature the double stranded DNA in the gel. The gel was briefly rinsed in distilled water and immersed in neutralisation buffer (0,5 mol/L Tris-HCl; 3 mol/L NaCL) twice for 15 minutes at room temperature. A parafilm was placed onto the porous metal support of the vacuum apparatus and screwed into position. A template slightly smaller than the gel was cut in the centre of the parafilm. A piece of Whatman 3 mm filter paper and Hybond N + nylon membrane (Amersham, Life Science, England) cut to the size of the template, was presoaked in sterile distilled water and placed overlapping the template, with the membrane above the filter paper. The gel was placed in contact with the nylon membrane and constantly covered with 20 x SSC buffer (3 mol/L NaCl; 0,3 mol/L sodium citrate). The DNA was transferred onto the nylon membrane by applying a vacuum of 10 cm of mercury evenly over the entire surface for 45 minutes.

After transfer the DNA was cross linked to the membrane by placing it for 3 minutes under UV light at 254 nm. The blotted gel was examined under UV light to ensure complete transfer of DNA. The membranes were air dried and stored at 4° C for hybridisation.

5.2.5.4 Hybridisation conditions

The membranes were prehybridised in a container with 20 mls prehybridisation solution (1% blocking reagent, 0,1 mol/L maleic acid, 0,15 mol/L NaCl) per 100cm^2 of membrane surface area at 68^0 C for one hour. The DIG-labelled probe was diluted to an optimal concentration of 10 pmol/ml in 3,5ml of standard hybridisation solution (5 x SSC, 1% blocking reagent, 0,1% N-lauroylsarcosine [w/v], 0,02% SDS [w/v]) for a 10 x 10 cm blot. The membranes were placed in hybridisation bags and replaced with standard hybridisation solution and diluted probe. The membranes were hybridised for three hours at 68° C with constant mixing of the hybridisation solution. At the end of hybridisation, the solution was removed from the bag and the membranes washed twice, 5 minute per wash, in 2 x wash

solution (2 x SSC, 0,1% SDS) at room temperature to remove any unbound probe. Thereafter the membranes were washed twice, 15 minute per wash, in 0,1 x wash solution (0,1 x SSC, 0,1% SDS) at 68^0 C.

5.2.5.5 Detection of probed fragments

The immobilised DNA fragments on the nylon membranes were detected with the Nucleic acid detection kit (Boehringer Mannheim) which allows the colorimetric detection of DIG labelled nucleic acids by using NBT (nitroblue tetrazolium) and X - Phosphate (BCIP) (50mg/ml 5-bromo-4-chloro-3-indolyl phosphate, toluidinium salt in 100% dimethylformamide) colour substrate. The membranes were briefly washed for one minute in buffer 1 (0,1 mol/L maleic acid, 0,15 mol/L NaCl) and 0,3% Tween 20 (w/v) and thereafter incubated for 30 minutes in 100 ml of buffer 2 (1% blocking reagent, 0,1 mol/L maleic acid, 0,15 mol/L NaCl). The anti-digoxigenin-alkaline phosphatase conjugate was diluted to 150 mU/ml (1:5000) in buffer 2 and the membranes incubated for 30 minutes with 20 ml of diluted antibody conjugate solution. Unbound antibody conjugate was removed by washing twice, for 15 minutes with 100 ml of buffer 1. The membranes were equilibrated for 2 minutes with 20 ml of buffer 3 (100 mM tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9,5). The membranes were incubated with freshly prepared colour-substrate (200 µl NBT/BCIP stock solution in 10 ml buffer 3) in a sealed plastic bag in the dark without shaking. When there was sufficient colour development observed in the DNA fragments, the reaction was stopped by washing the membranes for 5 minutes with 50 ml of buffer 4 (10 mM Tris-HCl, 1 mM EDTA, pH 8,0).

5.2.6 SEQUENCING OF 16S rDNA

TA cloning has a great advantage of generating a large amount of DNA suitable for sequencing. Both T7 and M13 sequencing primers are used to initiate the sequencing reaction close to the polylinker, and all sequences have this start in common but differ in the direction of the insertion of the fragment. In addition the sequence of the recombinant clones containing the insert would reveal the additional T:A base pair between the vector and insert DNA. Sequencing was performed using the automated Alf™ DNA sequencer

(Pharmacia LKB Biotechnology, USA) and on an ABI Prism 377 automated sequencer (Applied Biosystems, USA).

5.2.6.1 Preparation of plasmid DNA for sequencing

To ensure high quality and yields of plasmid DNA the Qiagen Plasmid Maxi kit (Qiagen Inc., USA) was used. The bacterial cultures containing plasmid DNA were grown in 100 millilitres of LB broth in the presence of ampicillin (Smith, Kline and Beechams Pharmaceuticals, UK) (50μg/ml) at 37^{0}C for 16 hours. The bacterial cells were harvested by centrifugation at 4^{0}C for 15 minutes at 10 000 rpm. All traces of the supernatant was removed. The bacterial pellet was resuspended in buffer P1 (100μg/ml RNase A; 50 mM Tris/HCL; 10 mM EDTA, pH 8.0). Ten millilitre of lysis buffer 2 (200 mM NaOH; 1% SDS) was added, mixed gently and incubated at room temperature for 5 minutes, after which ten millilitres of chilled neutralisation buffer 3 (3M K acetate , pH 5.5) was added, mixed immediately but gently, and incubated on ice for 20 minutes. The tubes were centrifuged at 4^{0}C for 30 minutes at 16 000 rpm. The supernatant was removed promptly and applied to a Qiagen tip 500 which had been equilibrated with 10 mls of buffer QBT (750 mM NaCl; 50 mM MOPS; 15% ethanol; pH 7.0; 0.15% Triton X - 100). The supernatant was allowed to enter the resin by gravity flow.

The Qiagen tip was washed twice with 30 mls each of wash buffer QC (1.0 M NaCl, 50 mM MOPS; 15% ethanol, pH 7.0). The plasmid DNA was eluted with 15 mls buffer QF (1.25 M NaCl; 50 mM Tris/HCl); 15% ethanol, pH 8.5) and precipitated with 0,7 volumes of isopropanol at room temperature. The tubes were centrifuged at 16 000rpm at 4°C for 30 minutes. The supernatant was removed carefully and the DNA washed with 15 mls of 70% cold ethanol, air dried and redissolved in 500μl of 1x TE buffer.

5.2.6.2 Purification of template DNA for automated sequencing

For successful automated DNA sequencing, pure template was essential. The number of bases which could be read accurately was directly related to the quality of the template DNA. Generally the DNA should be free from protein, RNA, chromosomal DNA and

organic solvents. The purification and concentration of the double - stranded DNA templates were done by applying 500µl of DNA sample onto Microcon 100 micro concentrator (Amicon Corporation, USA) which employs Amicon's low binding, anisotropic, hydrophilic YM membrane. This membrane has the ability to retain double stranded DNA molecules above 125 nucleotides, removing low molecular weight contaminants. After low speed centrifugation the retentate was transferred to a clean microfuge tube and the concentration of DNA re-estimated using Genequant (Pharmacia LKB Biotechnology).

5.2.6.3 Sequencing primers

Primers as shown in table XIII were used as sequencing primers. Internal "walking in primers" were designed and used subsequently.

5.2.6.4 Sequencing methods

5.2.6.4.1 *Automated Alf™ DNA sequencing method*

Sequencing of the recombinant clones containing the insert was performed by using the Autoread™ sequencing kit which is designed for use with the automated Alf™ DNA sequencer (Pharmacia LKB Biotechnology, USA) based on the standard dideoxysequencing method (Sanger *et al*, 1977). The method employed a non - radiochemical approach to sequencing. The primer was labelled with fluorescein at its 5'- terminus and annealed to the template. The fluorescent primers were extended by T7 DNA polymerase in four separate dideoxy reactions (A - adenosine, C - cytosine, G - guanine and T - thymine), creating four separate populations of fluorescently labelled chain - terminated fragments. The reactions were loaded into four adjacent lanes on a sequencing gel and electrophoresed. As the DNA fragments in each lane migrated through the gel, they passed a fixed laser beam, causing the generation of fluorescent signals which were then detected and stored in a computer for sequence determination and analysis.

TABLE XIII: NUCLEOTIDE SEQUENCES OF SEQUENCING PRIMERS

Name	Nucleotide Sequence
T7	5'- TAATACGACTCACTATAGGG - 3'
M13	5'- GTTTTCCCAGTCACGAC - 3'
M13 (reverse primer)	5'- TTCACAGGAAACAG -3'
CF308 (reverse custom primer)	5'- CCGAACCGCTGGCAACAA - 3'
CR289 (custom primer)	5'- TAGTAGGTGGGGTAACGGCT -3'

5.2.6.4.2 *Automated ABI Prism 377 sequencing method*

All sequencing expsriments were repeated and verified on an ABI Prism 377 Automated sequencer by Genomis Inc (USA). This is a non - radioactive approach to sequencing using ABI Prism cycle sequencing dye terminator (DT). The ready reaction kits containing amplitaq DNA polymerase (FS) were used according to the manufacturers instructions. Each 2', 3' - dideoxynucleoside - 5' - triphosphate nucleotide analog (ddNTP) is tagged with a different fluorescent label so that the ABI Prism instrument recognises the distinct label colours to give complete sequence information. In addition, since a different fluorescein label is used for each ddNTP, they can all be combined in a single reaction mixture, simplifying reaction set up considerably. The fluorescent dyes used are : 6-carboxy-2', 7'-dimethoxy-4', 5' dichlorofluorescein (JOE - green/A), 6-carboxyfluorescein (6-FAM - blue/C), N,N,N',N'-tetramethyl-6- carboxyrhodamine (TAMRA - yellow/G), 6-carboxy-X-rhodamine (ROX -red/T). Primers used were standard sequencing primers eg. M13 forward or reverse, T7 or custom primers (table XII). Cycle sequencing is the name given to the process of using repeated cycles of thermal denaturation, primer annealing and polymerisation to produce greater amounts of product in a DNA sequencing reaction. This amplification process employs a single primer so the amount of product DNA increases linearly with a number of cycles (McMahon *et al,* 1987). Cycle sequencing reactions were carried out in a Perkin Elmer 9600 Geneamp PCR system and electrophoresed on 36 cm, 5% Longranger (FMC BioProducts, USA) gel in a 2XA, thin run module on the ABI Prism 377 sequencer and detected by the laser beam.

5.2.6.5 Sequencing experiments

5.2.6.5.1 *Sequencing Reaction using the Autoread™ sequencing kit (Pharmacia LKB Biotechnology, USA)*

The sequencing gel consisted of urea (19g), water (24 mls), 10x TBE (7,5 mls), and Longranger gel (FMC BioProducts, USA) (6,0mls). All reagents were stirred slowly for 30 minutes at 40^0C and filtered through 0,45µm Millex HV vinyl filter. Polymerisation was obtained by the addition of 25µl of TEMED and 250µl 10% ammonium persulphate

to 50 ml of gel mix and mixed by gentle rotation. The plate was poured by expelling the gel mix at a steady pressure ensuring no bubbles were formed between the plates. The plate was allowed to polymerise for 2,5 hours.

Six recombinant DNA clones from each source ie. monocyte co-culture, frozen and formalin fixed paraffin wax embedded tissue biopsy specimens were sequenced (thrice) in both directions to rule out any nucleotide misincorporations which might have occurred during the PCR and the cloning step. Sequencing in both directions also verified and resolved some of the bases in regions where ambiguities occurred.

For sequencing, template DNA was used at a concentration of 0,05 to 0,2 pmoles and annealed to 2,0 pmoles of the respective sequencing primer.

Denaturing and annealing

In a 0,5 ml eppendorf tube, 10µl of template DNA, 2µl of unlabelled sequencing primer and 1,5µl of 1M NaOH was added and gently mixed and placed at 370^{0}C for 4 minutes.

Neutralisation and labelling

1,5µl of 1M HCl and 2µl of annealing buffer was added and gently mixed and placed at 37^{0}C for 10 minutes. Thereafter 1µl of label mix and 1µl of T7 DNA polymerase was added and incubated at 37^{0}C for 10 minutes.

Preparation of termination mixes

A, C, G, T were prepared as follows:

To 3µl of each respective termination mixes, 1µl of DMSO was added and stored on ice.

Termination reaction

1µl of extension buffer was added to each reaction and divided into 4 x 3,5µl in microtitre plates.

4µl of each respective A, C, G and T were added and incubated at 40^{0}C for 10 minutes. At the end of the incubation time, the reaction was stopped with 4µl of stop solution.

Each reaction was heat denatured at 85^{0}C for 4 minutes and 7µl was loaded onto the sequencing gel and electrophoresed using 0.8% TBE running buffer under the following conditions: 1900 volts; 65mA; 35w at a temperature of 45^{0}C to 48^{0}C for 600 minutes.

5.2.6.5.2 *Sequence reaction using Taq FS cycle sequencing kit (Perkin Elmer Corporation, USA)*

Reaction mixture

In a PCR reaction tube 4µl of water, 6µl of terminator ready reaction mix, 3µl of template DNA and 2µl of 6µM primer was added for each reaction and overlayed with 25µl of mineral oil.

The tubes were placed in the thermal cycler for the following cycles :

Stage 1 (1 cycle)

step 1: 95^0 for 1:00 minute

Stage 2 (30 cycles)

step 1: 95^0 for 0:30 minute

step 2: 40^0 for 0:30 minute

step 3: 60^0 for 3:00 minutes with a ramp of 2sec/^{0}C.

Purification of extension product

After completion of the sequencing reaction, the extension products were transferred (15µl) to a 1,5 ml microcentrifuge tubes. 55,5µl of 70% ethanol with 0,5 mM $MgCl^2$ was added to the sequencing reaction, briefly vortexed and left at room temperature for 10 minutes to allow the precipitation of the extension products. The samples were centrifuged for 15 minutes at room temperature. The supernatant was carefully aspirated and the pellet was dried in a vacuum centrifuge for 5 minutes. The extension products were denatured for 2 minutes in a boiling water bath and quenched on ice for 1 minute. The samples were transferred to an autosampler tube with a septum and loaded on the ABI Prism genetic analyser for analysis.

5.2.6.6 Sequence alignment and phylogenetic analyses

All symbols used are those as recommended by the International Union of Pure and Applied Chemistry - International Union of Biochemistry (IUPAC - IUB) Biochemical nomenclature Commission. The sequences were aligned using Autoassemble 1.3 from ABI using a 50 base overlap and a 10% mismatch.

Nucleotide sequences obtained in this study were compared to the most similar 16S rRNA sequences identified from the GenBank database and Ribosomal Databse Project (Maidak *et al,* 1994). To this group of sequences, the 16S rRNA sequences from *Neisseria* (Gram negative, beta subgroup), *Rhizobium* (Gram negative, alpha subgroup), and *Caulobacter* (Gram negative, alpha subgroup), and *Bacillus subtilis* (Gram positive, low G+C subgroup) were added. The 16S rDNA sequences of all sequences were aligned and compared to determine the similarity. The multiple alignment of the sequences was performed by using DNASIS V2.1 (Hitachi Software, California) based on the Higgins and Sharp algorithm (Higgins and Sharp, 1988) and MEGA (molecular evolutionary genetics analysis) (Kumar *et al*, 1993) computer programme software packages. The final aligned sequence consisted of 1380 base pairs including alignment gaps.

The phylogenetic relationships were inferred by a number of methods. These were the unweighted pair-wise grouping method of arithmetic average (UPGMA) (Tanimura *et al*, 1985) and by the neighbor-joining method (Saitou and Nel; 1987) using Tajima and Nei distances with 500 bootstraps (Felsenstein, 1985), Heuristic maximum parsimony, branch and bound maximum parsimony from MEGA (Saitou and Nel; 1987; Kumar *et al*, 1993), DNA parsimony with 100 bootstraps and DNA Penny from PHYLIP (phylogenetic inference package) (Felsenstein, 1989).

5.2.6.7 Nucleotide sequence accession numbers

Previously published 16S rRNA sequences of *Escherichia coli* A14565; *Acyrthosiphon pisum symbionts* M27040; *Enterobacter species* U39556; *Erwinia amylovora* X83265; *Citrobacter freundii* M59291; *Erwinia carotovora* M59149; *Serratia marcescens* M59160; *Yersinia enterocolitica* Z49829; *Yersinia aldovae* X75277; *Klebsiella pneumoniae* X87276; *Hafnia alvei* M59155; *Salmonella typhimurium* U90316; *Rhizobium leguminosarum* X67233; *Neisseria gonorrhoeae* X07714; *Caulobacter crescentas* M83799; *Bacillus subtilis* X60646 were added to the analysis.

5.3 RESULTS

5.3.1 GENOMIC DNA EXTRACTION

The DNA extracted from three different monocyte co-cultures, uninfected monocytes, frozen, paraffin wax embedded tissue biopsy specimens and tumour biopsy specimen gave identical results. Although a little more time consuming, the phenol-chloroform extraction procedure was included for all specimens to prevent any inhibitory substances which might have been present and interfere with the PCR. The DNA from most of the three sets of specimens was of high molecular weight and showed little degradation as shown in figure 63.

5.3.2 AMPLIFICATION OF 16S rDNA

PCR amplification of the 16S rRNA gene using universal bacterial primers amplified a fragment of approximately 1380 base pairs (bp) from the DNA obtained from monocyte co-cultures, frozen and paraffin wax embedded biopsy specimens (Figure 64). No fragments were amplified from the non-infected monocytes or the negative water control. A 1380 bp PCR product was obtained after amplification and detected from laboratory cultures of *Escherichia coli* and *Staphylococcus aureus* (Figure 64). Similar results were obtained with laboratory strains of *Serratia marcescence, Klebsiella pneumoniae, Klebsiella oxytoca* and *Acinetobacter anitratus.*

5.3.3 CLONING OF AMPLIFIED FRAGMENTS

T:A cloning was efficient for the cloning of the 1380 bp PCR product from the monocyte co-cultures, frozen and paraffin wax embedded tissue biopsy specimens into pMOS*blue* T - vector. The plasmid DNA extracted by the alkaline lysis method from the recombinant clones are shown in figure 65. The recombinant clones were restricted with *Bam*HI and *Pst*I to confirm the presence of the insert before sequencing. A 1380bp fragment was clearly seen upon enzyme restriction in all recombinant clones from the different sources mentioned above as depicted in figure 66. The DNA from these recombinant clones was subsequently used in sequencing experiments.

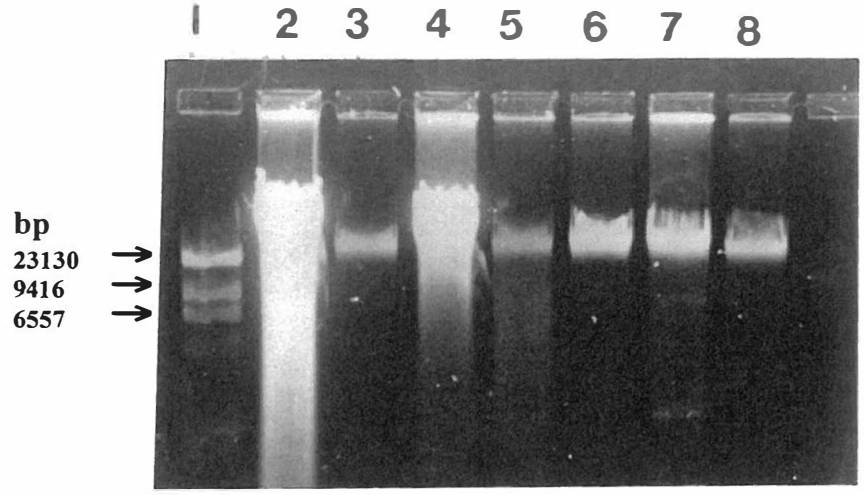

Figure 63: Agarose (1%) gel electrophoresis showing genomic DNA.
Lane 1- Lamda DNA cleaved with restriction endonuclease *Hind*III ; Lane 2 - monocyte co-culture; Lane 3 - monocyte co-culture; Lane 4 -frozen tissue biopsy specimen; Lane 5 - paraffin wax embedded tissue biopsy specimen; Lane 6 - uninfected monocytes; Lane 7 - monocyte co-culture; Lane 8 - breast tumour biopsy specimen (digestion control).

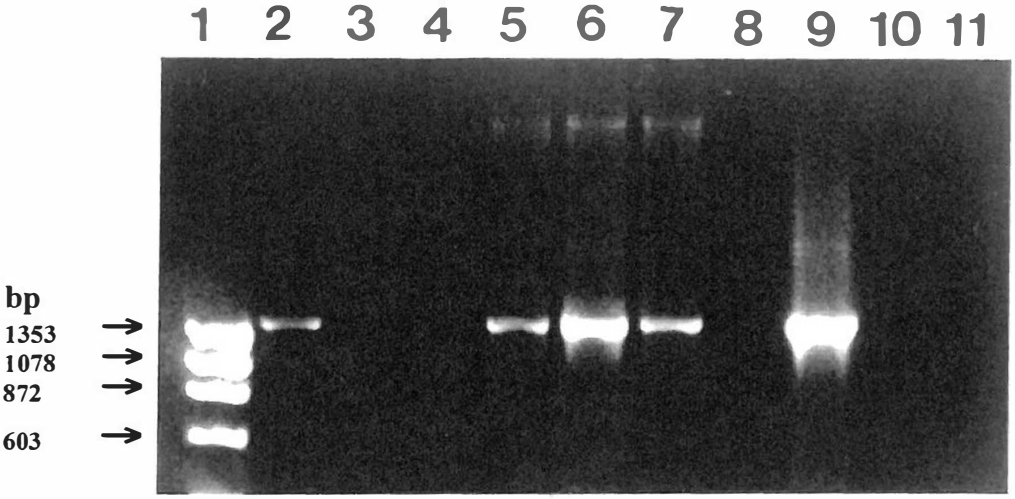

Figure 64: Agarose (1,5%) gel electrophoresis of PCR amplification products. These products were generated with the universal bacterial primers P1F and P4R which were designed to amplify~1380 base pair fragment of the 16S rRNA gene. Lane 1 -Molecular weight marker ϕX174 DNA cleaved with restriction endonuclease *Hae*III; Lane 2 - *Escherichia coli* (positive amplification control); Lane 3 - uninfected monocytes (negative amplification control); Lane 4 - negative water PCR control; Lane 5 - monocyte co-cultures; Lane 6 - paraffin wax embedded biopsy specimen; Lane 7 -frozen biopsy specimen; Lane 8 - breast tumour biopsy specimen (negative bacterial amplification control); Lane 9 - *Staphylococcus aureus* (positive amplification control).

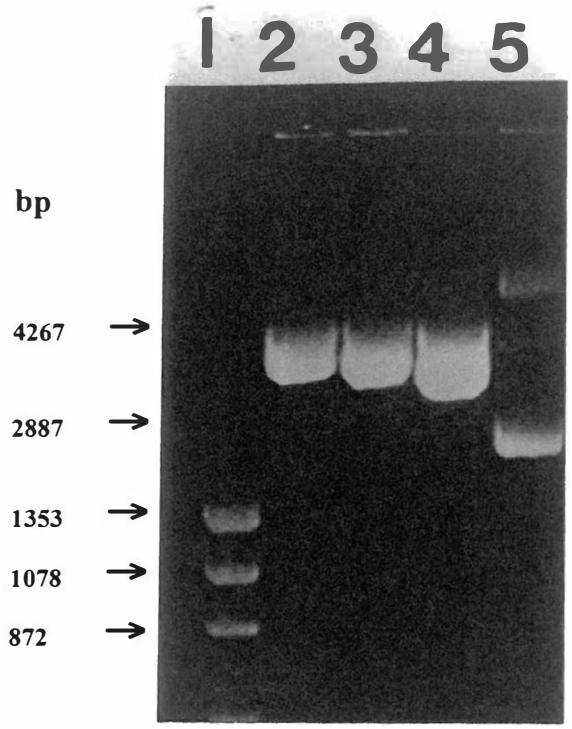

Figure 65: Ligation reactions of T:A cloning. Plasmid DNA from recombinant clones and non - recombinant clones was extracted and electrophoresed on 1,5% agarose gel for the presence of inserts. Lane 1 - Molecular weight marker ϕX174 DNA cleaved with restriction endonuclease *Hae*III; lane 2 , 3, 4 - recombinant clone (vector + insert = 4267bp); 2 - monocyte co-culture (KH 6), lane 3 - frozen tissue biopsy specimen - KH 6, and 4 - formalin fixed paraffin wax tissue biopsy specimen (KH 34); lane 5 non - recombinant clone (vector alone - 2887bp; figure 62).

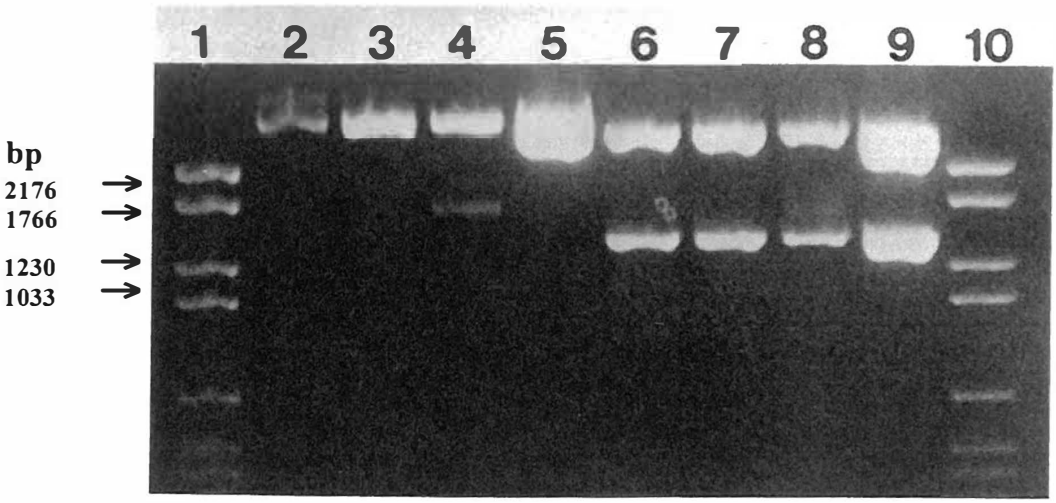

Figure 66: Agarose (1,5%) gel electrophoresis showing restriction analysis of recombinant clones with *Bam*HI and *Pst*I. Lanes 1 and 10 - fragments pbR 328 DNA cleaved with restriction enzyme *Bg*I and *Hinf*I; lane 2, 3, 4 and 5 - unrestricted DNA from monocyte co-cultures (2 and 3), frozen tissue biopsy specimen (4) and paraffin wax embedded biopsy specimen (5). Lanes 6,7,8 and 9 - restricted clones corresponding to unrestricted clones, showing inserts of approximately 1380 bp.

5.3.4 HYBRIDISATION

The universal bacterial probe RDR 245 has been designed from a conserved region of the 16S rRNA gene which is located at bp 1369 to 1395 (antisense) of the *E. coli* gene. The amplified 1380 bp PCR products obtained using template DNA from bacterial cultures with P1F and P4R primers as well as the recombinant clones restricted with *Bam*HI and *Pst*I were run on agarose gels and blotted onto nylon membranes and hybridised with DIG labelled RDR245. All 1380 bp PCR products obtained from bacterial culture DNA and the restricted fragments from the recombinant clones hybridised with the probe with the intensity of the signal varying roughly to the amount of DNA present on the blot. The agarose gel and the hybridised blot are shown in figures 67 and 68.

The DIG labelled Gram negative probe DLO4 hybridised to the monocyte co-culture and tissue biopsy specimens DNA as well as the 1380 bp amplified fragments obtained from Gram negative bacterial cultures, but not to those from Gram positive organisms (*S. aureus*) (Figures 69 and 70). The DIG labelled universal Gram positive probe (RWO3) tested, specifically detected amplicons obtained from Gram positive organisms (*S. aureus*) and was negative with all the amplicons from Gram negative organisms as well as the restricted fragments (1380bp) obtained from the recombinant clones from monocyte co-culture and tissue biopsy specimens (Figure 71 and 72). The hybridisation with probes CF308 and CR289 which were designed to be *C. granulomatis* specific is shown in figures 73, 74, 75 and 76). These probes showed reactions with the restricted fragments obtained from the recombinant clones from monocyte co-culture and tissue biopsy specimens. Weak reactions were also evident with *K. pneumoniae*, *K. oxytoca* and *S. marcescence*.

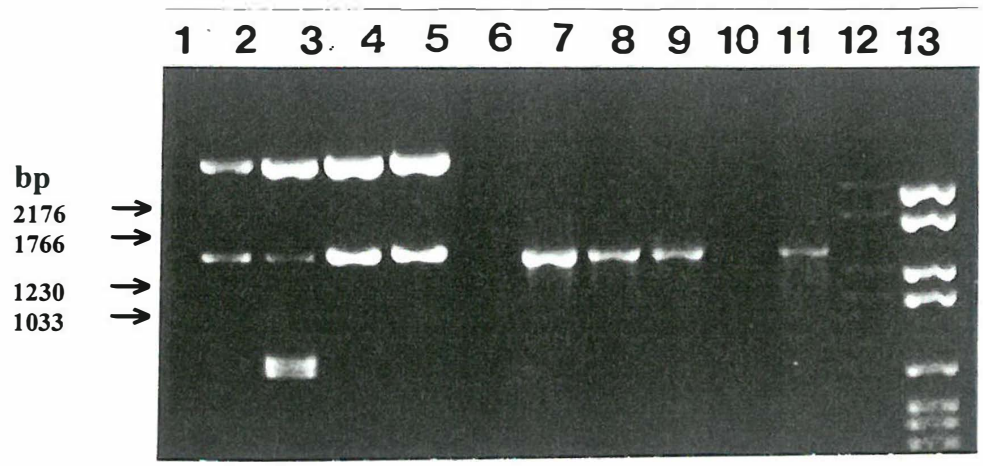

Figure 67: Agarose gel (1%) electrophoresis of restricted recombinant clones and amplified products obtained from bacterial DNA for hybridisation with universal bacterial probe RDR245. Lanes 1 and 12 - DIG labelled fragments of pbR 328 DNA cleaved with restriction enzyme *Bg*I and *Hinf*I; lanes 2, 3 -frozen biopsy specimen, lane 4 - monocyte co-culture, lane 5 - paraffin wax embedded biopsy specimen; lane 6 - empty; 7 - *E. coli*; lane 8 - *K. pneumoniae*; lane 9 -*K. oxytoca*; lane 10 - negative PCR water control; lane 11 - *S. aureus*.

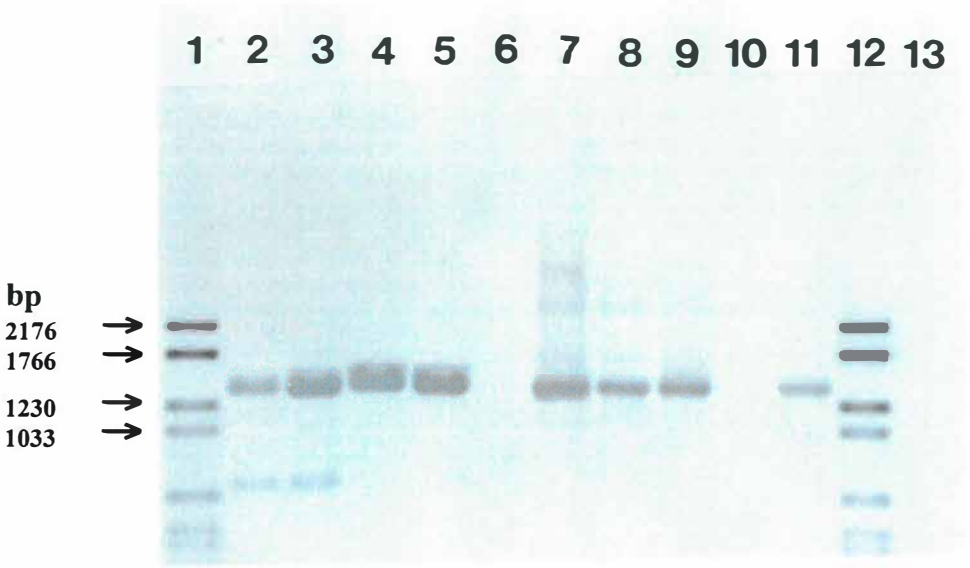

Figure 68: Hybridisation of the corresponding restricted recombinant clones and amplified products from bacterial DNA with DIG labelled universal bacterial probe RDR245 as depicted in figure 67.

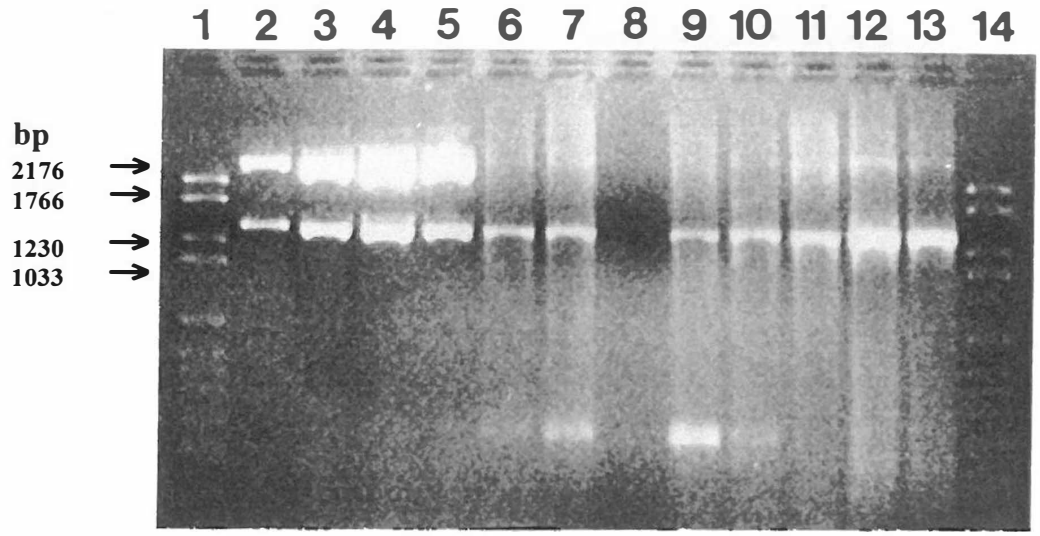

Figure 69: Agarose gel (1%) electrophoresis of restricted recombinant clones and products from amplified bacterial DNA for hybridisation with Gram negative bacterial probe DLO4. Lanes 1 and 14 - DIG labelled fragments of pbR 328 DNA cleaved with restriction enzyme *Bg*I and *Hinf*I; lanes 2, 3 - frozen biopsy specimen, lane 4 - monocyte co-culture, lane 5 - paraffin wax embedded biopsy specimen; lane 6 - *E. coli*; lane 7 - *K. pneumoniae*; lane 8 - negative PCR water control; lane 9 - *K. oxytoca*; lane 10 - *S. marcescence*; lane 11 - *A. anitratus*; lane 12 - *S. aureus*; lane 13 - *S. aureus*.

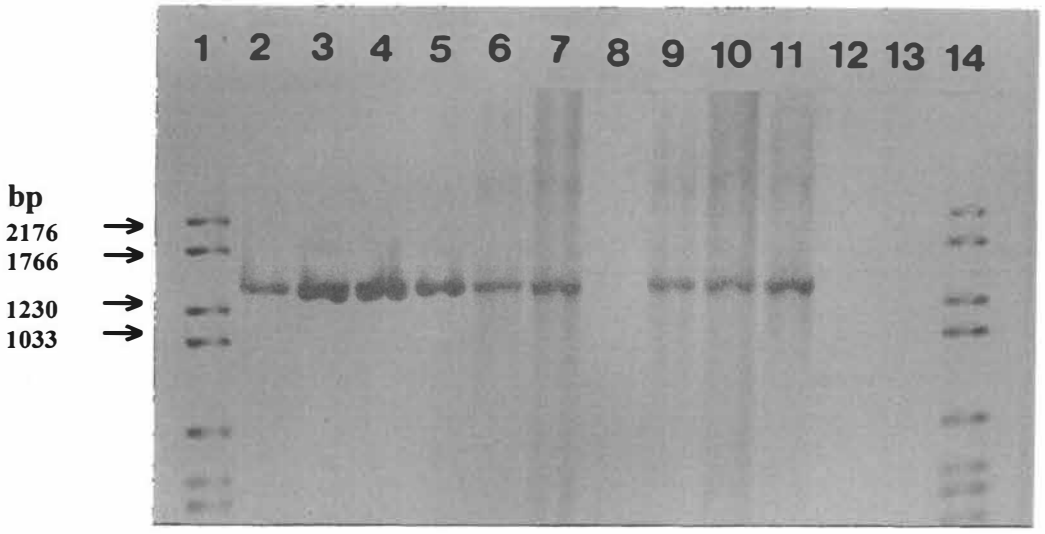

Figure 70: Hybridisation of the corresponding restricted recombinant clones and amplified products from bacterial DNA with DIG labelled Gram negative bacterial probe DLO4 as depicted in figure 69.

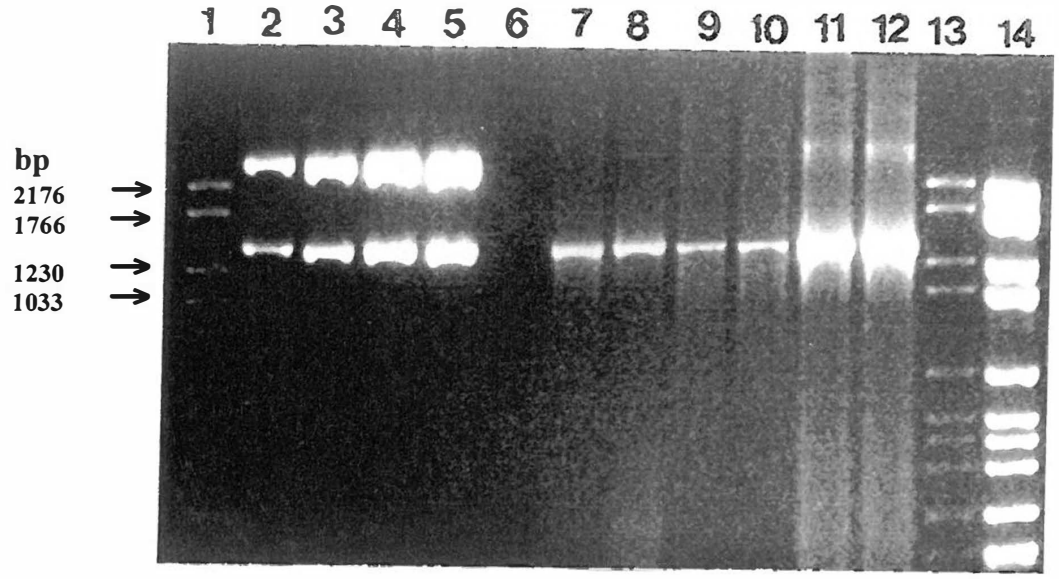

Figure 71: Agarose gel (1%) electrophoresis of restricted recombinant clones and amplified products from bacterial DNA for hybridisation with Gram positive bacterial probe RWO3. Lanes 1 and 13 - DIG labelled fragments of pbR 328 DNA cleaved with restriction enzyme *Bg*I and *Hinf*I; lanes 2, 3 - frozen biopsy specimen, lane 4 - monocyte co-culture, lane 5 - paraffin wax embedded biopsy specimen; lane 6 - negative PCR water control; lane 7 - *E. coli*; lane 8 - *K. pneumoniae*; lane 9 - *K. oxytoca*; lane 10 - *S. marcescence*; lane 11 - *S. aureus*; lane 12 - *S. aureus*.

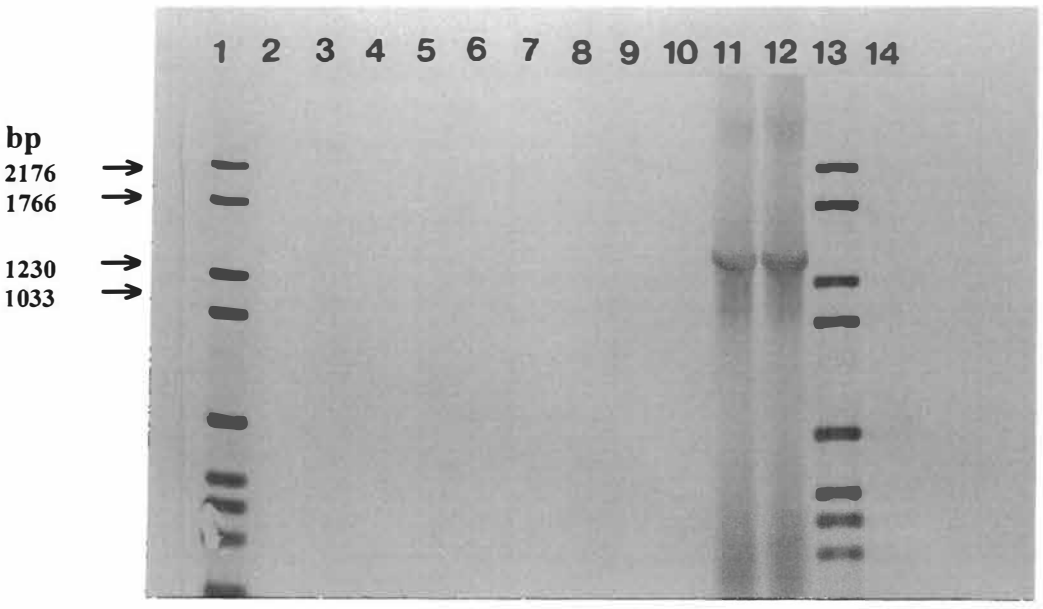

Figure 72: Hybridisation of the corresponding restricted recombinant clones and amplified products from bacterial DNA with DIG labelled universal Gram positive probe RWO3 as depicted in figure 71.

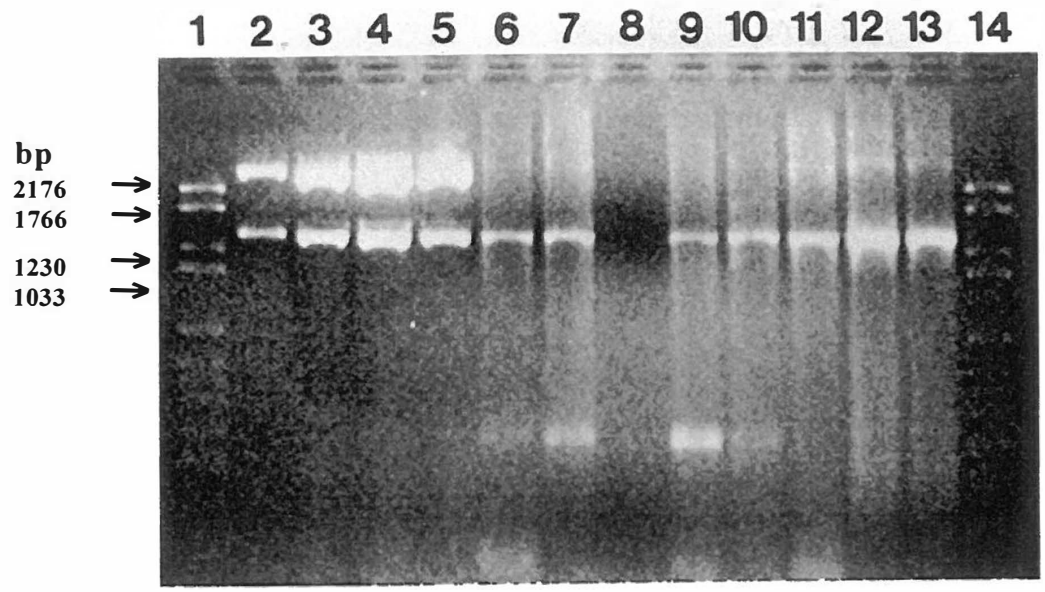

Figure 73: Agarose gel (1%) electrophoresis of restricted recombinant clones and amplified products from bacterial DNA for hybridisation with probe CF 308. Lanes 1 and 14 - DIG labelled fragments of pbR 328 DNA cleaved with restriction enzyme *Bg*I and *Hinf*I; lanes 2, 3 - frozen biopsy specimen, lane 4 - monocyte co-culture, lane 5 - paraffin wax embedded biopsy specimen; lane 6 - *E. coli*; lane 7 - *K. pneumoniae*; lane 8 - negative PCR water control; lane 9 - *K. oxytoca*; lane 10 -*S. marcescence*; lane 11 - *A. anitratus*; lane 12 - *S. aureus*; lane 13 - *S. aureus*.

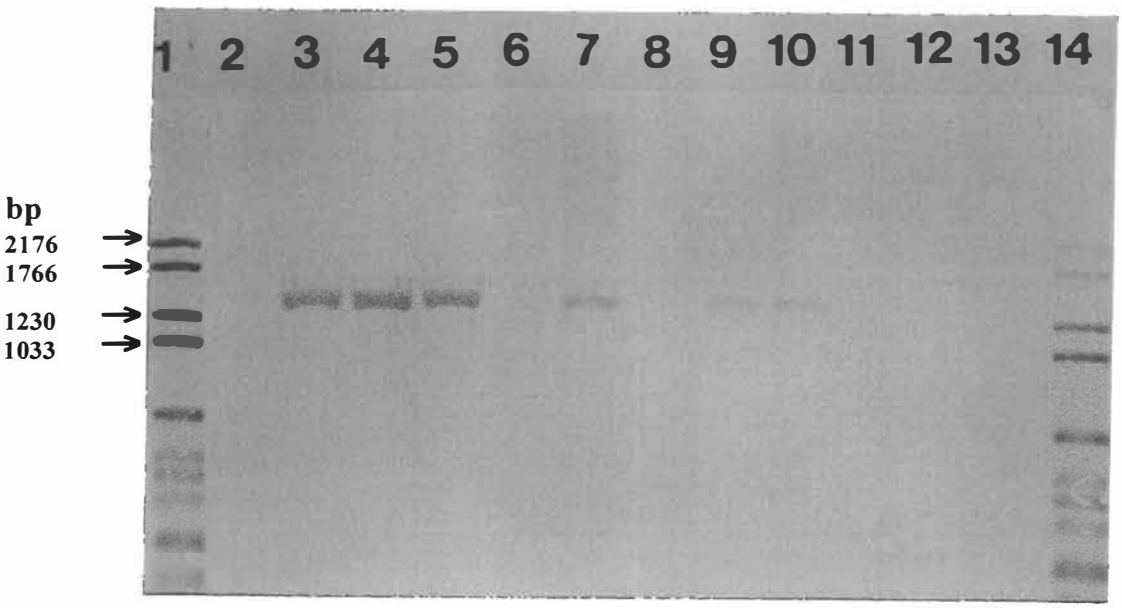

Figure 74: Hybridisation of the corresponding restricted recombinant clones and amplified products from bacterial DNA with DIG labelled probe CF308 as depicted in figure 73.

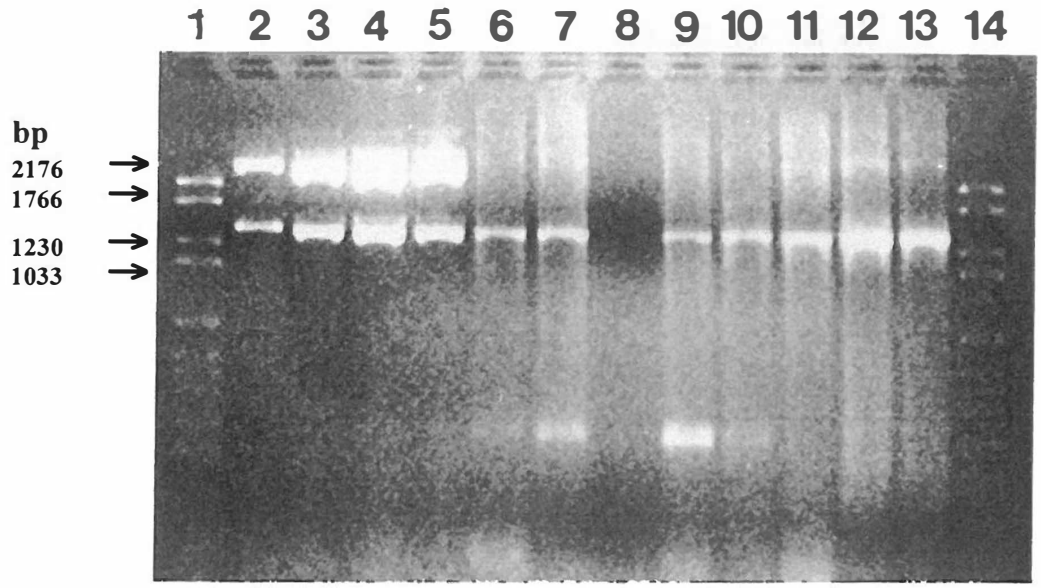

Figure 75: Agarose gel (1%) electrophoresis of restricted recombinant clones and amplified products from bacterial DNA for hybridisation with probe CR289. Lanes 1 and 14 - DIG labelled fragments of pbR 328 DNA cleaved with restriction enzyme *Bg*I and *Hinf*I; lanes 2, 3 - frozen biopsy specimen, lane 4 - monocyte co-culture, lane 5 - paraffin wax embedded biopsy specimen; lane 6 - *E. coli*; lane 7 - *K. pneumoniae*; lane 8 - negative PCR water control; lane 9 - *K. oxytoca*; lane 10 -*S. marcescence*; lane 11 - *A. anitratus*; lane 12 - *S. aureus*; lane 13 - *S. aureus*.

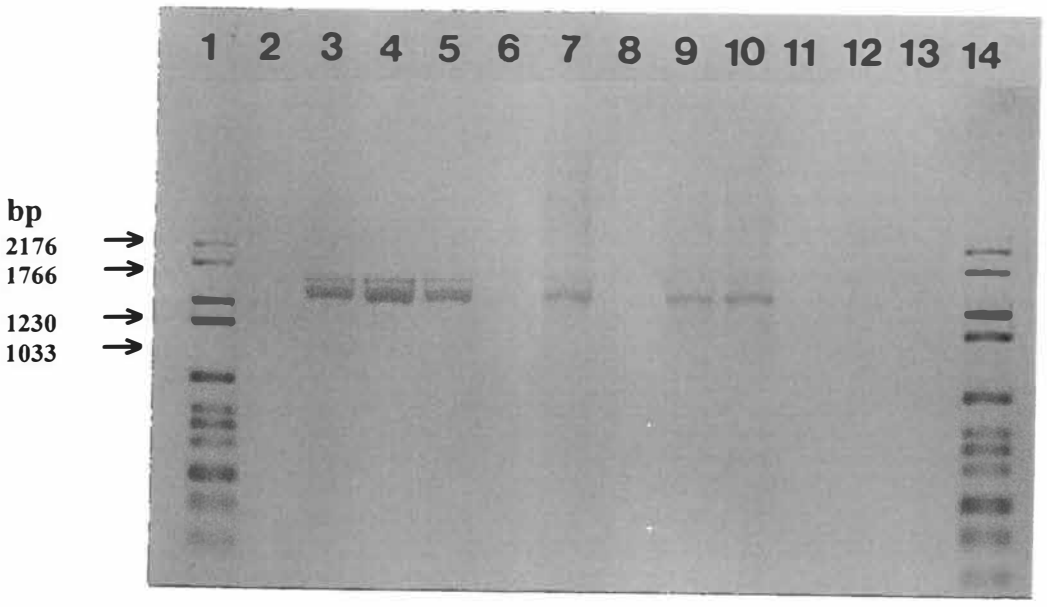

Figure 76: Hybridisation of corresponding restricted recombinant clones and amplified products from bacterial DNA with DIG labelled probe CR289 as depicted in figure 75.

5.3.5 SEQUENCE ANALYSIS

The sequence read outs of the recombinant clones of KH22 (monocyte co-culture); KH6 (frozen tissue biopsy specimen); and KH34 (formalin fixed paraffin wax embedded tissue biopsy specimen) with the corresponding alignment of the three sequences are shown in figure 77. The corresponding electropherograms and the homology data are included in the appendix section.

5.3.5.1 Comparison of 16S rRNA gene sequences

Since there are no strains available as references cultures, it was not possible to compare the sequences obtained to the sequences of reference strains. The nucleotide sequences of 1380 bases of 16S rRNA genes represents approximately 90% of the 16S rRNA gene of a single uncharacterised bacterium. The sequence data demonstrated that the sequences were identical. The level of DNA homology of the sequences of bacteria obtained from the monocyte co-cultured specimen, frozen and formalin fixed paraffin wax embedded biopsy specimen was >98% (Appendix I). The single nucleotide differences observed amongst the three sequences were at varying positions and are indicated in table XIV. These differences were evident on the electropherograms.

5.3.5.2 Sequence similarity analysis

The multiple sequence alignment and similarity of 16S rDNA to show DNA relatedness for strains of *C. granulomatis* from KH 22, KH 6 and KH 34 is shown in figure 77. To determine where to place the genus *Calymmatobacterium* within the gamma subclass of *Proteobacteria*, levels of similarty to other genera were calculated. The partial 16S rRNA sequences of KH 22, KH 6 and KH 34 were compared with previously determined 16S rRNA sequences of prokaryotes included in the GenBank as shown in table XV. KH 22, KH 6 and KH 34 exhibited 95% and 94% of sequence similarity with members of the *Klebsiella* and *Enterobacteria* species respectively, which was the highest level of similarity observed with any of the sequences included in the analysis. Significantly lower levels of relatedness were found for species belonging to the *Neisseria* and *Bacillus* groups.

```
                        10         20         30         40         50
22.SEQ       1 AGTTTGATCC TGGCTCAGAT TGAACGCTGG CGGCAGGCCT AACACATGCA  50
6.SEQ        1 AGTTTGATCC TGGCTCAGAT TGAACGCTGG CGGCAGGCCT AACACATGCA  50
34.SEQ       1 AGTTTGATCC TGGCTCAGAT TGAACGCTGG CGGCAGGCCT AACACATGCA  50
                        60         70         80         90        100
22.SEQ      51 AGTCGAGCGG TAGCACAGAG AGCTTGCTCT CGGGTGACGA GCGGCGGACG 100
6.SEQ       51 AGTCGAGCGG TAGCACAGAG AGCTTGCTCT CGGGTGACGA GCGGCGGACG 100
34.SEQ      51 AGTCGAGCGG TAGCACAGAG AGCTTGCTCT CGGGTGACGA GCGGCGGACG 100
                       110        120        130        140        150
22.SEQ     101 GGTGAGTAAT GTCTGGGAAA CTGCCTGATG GAGGGGGATA ACTACTGGAA 150
6.SEQ      101 GGTGAGTAAT GTCTGGGAAA CTGCCTGATG GAGGGGGATA ACTACTGGAA 150
34.SEQ     101 GGTGAGTAAT GTCTGGGAAA CTGCCTGATG GAGGGGGATA ACTACTGGAA 150
                       160        170        180        190        200
22.SEQ     151 ACGGTAGCTA ATACCGCATA ACGTCGCAAG ACCAAAGTGG GGGACCTTCG 200
6.SEQ      151 ACGGTAGCTA ATACCGCATA ATGTCGCAAG ACCAAAGTGG GGGACCTTCG 200
34.SEQ     151 ACGGTAGCTA ATACCGCATA ATGTCGCAAG ACCAAAGTGG GGGACCTTCG 200
                       210        220        230        240        250
22.SEQ     201 GGCCTCATGC CATCAGATGT GCCCAGATGG GATTAGCTAG TAGGTGGGGT 250
6.SEQ      201 GGCCTCATGC CATCAGATGT GCCCAGATGG GATTAGCTAG TAGGTGGGGT 250
34.SEQ     201 GGCCTCATGC CATCAGATGT GCCCAGATGG GATTAGCTAG TAGGTGGGGT 250
                       260        270        280        290        300
22.SEQ     251 AACGGCTCAC CTAGGCGACG ATCCCTAGCT GGTCTGAGAG GATGACCAGC 300
6.SEQ      251 AACGGCTCAC CTAGGCGACG ATCCCTAGCT GGTCTGAGAG GATGACCAGC 300
34.SEQ     251 AACGGCTCAC CTAGGCGACG ATCCCTAGCT GGTCTGAGAG GATGACCAGC 300
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22.SEQ     301 CACACTGGAA CTGAGACACG GTCCAGACTC CTACGGGAGG CAGCAGTGGG 350
6.SEQ      301 CACACTGGAA CTGAGACACG GTCCAGACTC CTACGGGAG- CAGCAGTGGG 350
34.SEQ     301 CACACTGGAA CTGAGACACG GTCCAGACTC CTACGGGAG- CAGCAGTGGG 350
                       360        370        380        390        400
22.SEQ     351 GAATATTGCA CAATGGGCGC AAGCCTGATG CAGCCATGCC GCGTGTGTGA 400
6.SEQ      351 GAATATTGCA CAATGGGCGC AAGCCTGATG CAGCCATGCC GCGTGTGTGA 400
34.SEQ     351 GAATATTGCA CAATGGGCGC AAGCCTGATG CAGCCATGCC GCGTGTGTGA 400
                       410        420        430        440        450
22.SEQ     401 AGAAGGCCTT CGGGTTGTAA AGCACTTTCA GCGGGGAGGA AGGCGATAAG 450
6.SEQ      401 AGAAGGCCTT CGGGTTGTAA AGCACTTTCA GCGGGGAGGA AGGCGTTAAG 450
34.SEQ     401 AGAAGGCCTT CGGGTTGTAA AGCACTTTCA GCGGGGAGGA AGGCGTTAAG 450
                       460        470        480        490        500
22.SEQ     451 GTTAATAACC TTGGCGATTG ACGTTACCCG CAGAAGAAGC ACCGGCTAAC 500
6.SEQ      451 GTTAATAACC TTGGCGATTG ACGTTACCCG CAGAAGAAGC ACCGGCTAAC 500
34.SEQ     451 GTTAATAACC TTGGCGATTG ACGTTACCCG CAGAAGAAGC ACCGGCTAAC 500
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                          510        520        530        540        550
22.SEQ   501 TCCGTGCCAG CAGCCGCGGT AATACGGAGG GTGCAAGCGT TAATCGGAAT 550
6.SEQ    501 TCCGTGCCAG CAGCCGCGGT AATACGGAGG GTGCAAGCGT TAATCGGAAT 550
34.SEQ   501 TCCGTGCCAG CAGCCGCGGT AATACGGAGG GTGCAAGCGT TAATCGGAAT 550
                          560        570        580        590        600
22.SEQ   551 TACTGGGCGT AAAGCGCACG CAGGCGGTCT GTCAAGTCGG ATGTGAAATC 600
6.SEQ    551 TACTGGGCGT AAAGCGCACG CAGGCGGTCT GTCAAGTCGG ATGTGAAATC 600
34.SEQ   551 TACTGGGCGT AAAGCGCACG CAGGCGGTCT GTCAAGTCGG ATGTGAAATC 600
                          610        620        630        640        650
22.SEQ   601 CCCGGGCTCA ACCTGGGAAC TGCATTCGAA ACTGGCAGGC TAGAGTCTTG 650
6.SEQ    601 CCCGGGCTTA ACCTGGGAAC TGCATTCGAA ACTGGCAGGC TAGAGTCTTG 650
34.SEQ   601 CCCGGGCTTA ACCTGGGAAC TGCATTCGAA ACTGGCAGGC TAGAGTCTTG 650
                          660        670        680        690        700
22.SEQ   651 TAGAGGGGGG TAGAATTCCA GGTGTAGCGG TGAAATGCGT AGAGATCTGG 700
6.SEQ    651 TAGAGGGGGG TAGAATTCCA GGTGTAGCGG TGAAATGCGT AGAGATCTGG 700
34.SEQ   651 TAGAGGGGGG TAGAATTCCA GGTGTAGCGG TGAAATGCGT AGAGATCTGG 700
                          710        720        730        740        750
22.SEQ   701 AGGAATACCG GTGGCGAAGG CGGCCCCCTG GACAAAGACT GACGCTCAGG 750
6.SEQ    701 AGGAATACCG GTGGCGAAGG CGGCCCCCTG GACAAAGACT GACGCTCAGG 750
34.SEQ   701 AGGAATACCG GTGGCGAAGG CGGCCCCCTG GACAAAGACT GACGCTCAGG 750
                          760        770        780        790        800
22.SEQ   751 TGCGAAAGCG TGGGGAGCAA ACAGGATTAG ATACCCTGGT AGTCCACGCC 800
6.SEQ    751 TGCGAAAGCG TGGGGAGCAA ACAGGATTAG ATACCCTGGT AGTCCACGCC 800
34.SEQ   751 TGCGAAAGCG TGGGGAGCAA ACAGGATTAG ATACCCTGGT AGTCCACGCC 800
                          810        820        830        840        850
22.SEQ   801 GTAAACGATG TCGATTTGGA GGTTGTGCCC TTGAGGTGTG GCTTCCGGAG 850
6.SEQ    801 GTAAACGATG TCGATTTGGA GGTTGTGCCC TTGAGGCGTG GCTTCCGGAG 850
34.SEQ   801 GTAAACGATG TCGATTTGGA GGTTGTGCCC TTGAGGCGTG GCTTCCGGAG 850
                          860        870        880        890        900
22.SEQ   851 CTAACGCGTT AAATCGACCG CCTGGGGAGT ACGGCCGCAA GGTTAAAACT 900
6.SEQ    851 CTAACGCGTT AAATCGACCG CCTGGGGAGT ACGGCCGCAA GGTTAAAACT 900
34.SEQ   851 CTAACGCGTT AAATCGACCG CCTGGGGAGT ACGGCCGCAA GGTTAAAACT 900
                          910        920        930        940        950
22.SEQ   901 CAAATGAATT GACGGGGGCC CGCACAAGCG GTGGAGCATG TGGTTAATTC 950
6.SEQ    901 CAAATGAATT GACGGGGGCC CGCACAAGCG GTGGAGCATG GGTTAATTC 950
34.SEQ   901 CAAATGAATT GACGGGGGCC CGCACAAGCG GTGGAGCATG TG-TTAATTC 950
                          960        970        980        990       1000
22.SEQ   951 GATGCAACGG GAGAC-TTAC TGTTTTGACA TCCACAGAAT TTCCAGAGAT 1000
6.SEQ    951 GATGCAACGG GAGACCTTAC TGTTTTGACA TCCACAGAAT TTCCAGAGAT 1000
34.SEQ   951 GATGCAACGG GAGACCTAAC TGTTTTGACA TCAACAGAAT TTCCAGAGAT 1000
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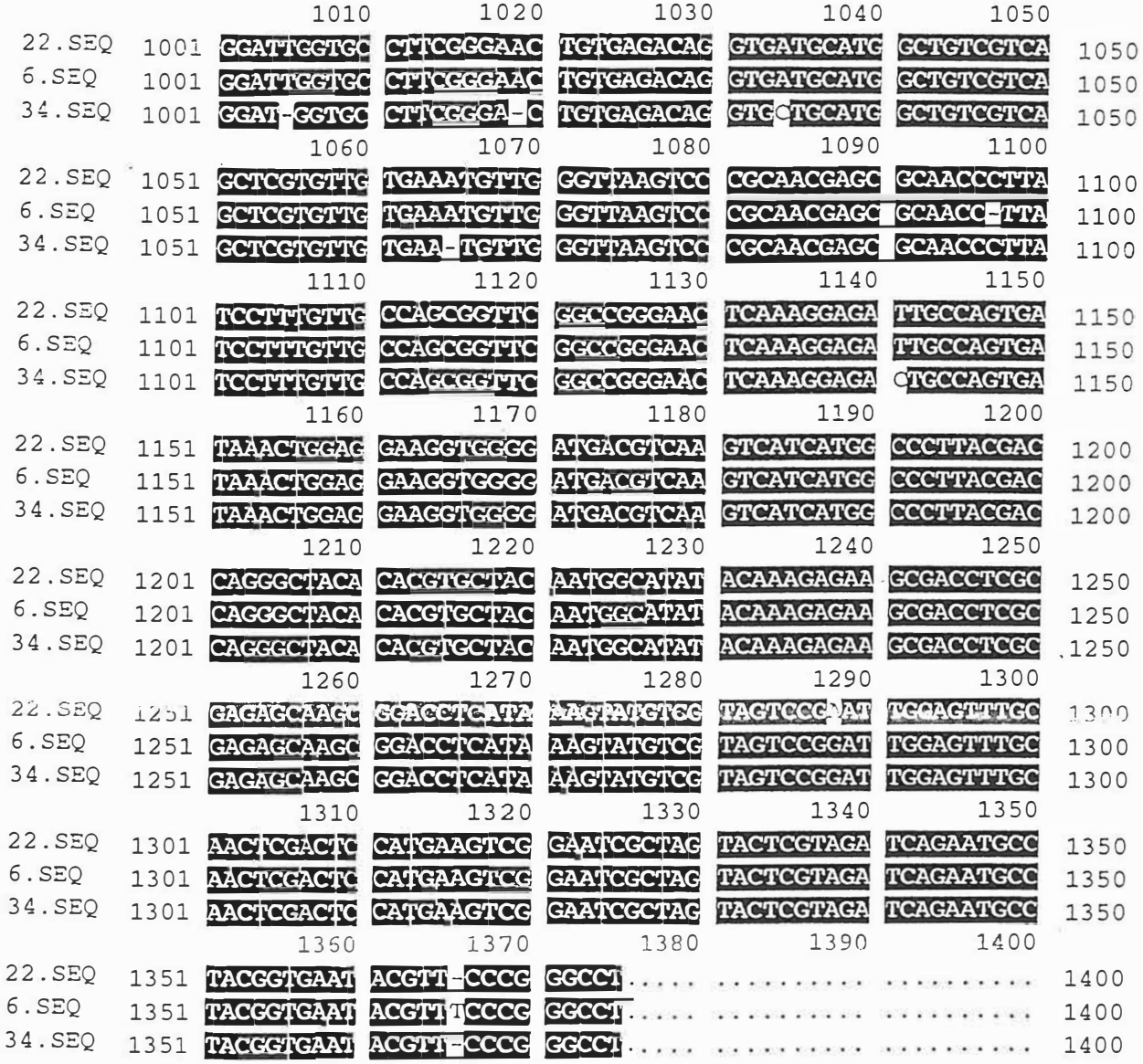


Figure 77: Multiple sequence alignment of 16S rDNA for strains of *C. granulomatis* of KH 22, KH 6 and KH 34. The region shown corresponds to positions 10 to 1390 of the *E. coli* 16S rRNA gene. Shaded area indicates similar bases, dashes (-) indicate alignment gaps.

TABLE XIV: 16S RIBOSOMAL DNA SEQUENCE DIFFERENCES DERIVED FROM BACTERIA FROM THE MONOCYTE CO-CULTURE SPECIMEN (KH 22), FROZEN (KH 6) AND FORMALIN FIXED PARAFFIN WAX EMBEDDED TISSUE BIOPSY SPECIMEN (KH 34)

16S rRNA position	Strain		
	KH 22	KH 6	KH 34
171	C	T	T
445	A	T	T
609	C	T	T
837	T	C	C
941	T	G	T
943	G	T	◆
966	◆	C	C
968	T	T	A
983	C	C	A
1005	T	T	◆
1019	A	A	◆
1034	A	A	C
1065	A	A	◆
1097	C	◆	C
1141	T	T	C
1288	A	G	G

◆ indicates deletion

TABLE XV: LEVELS OF 16S rDNA SEQUENCE SIMILARITIES BETWEEN *CALYMMATOBACTERIUM GRANULOMATIS* AND SOME REFERENCES SPECIES BELONGING TO ALPHA, BETA AND GAMMA SUBCLASS OF *PROTEOBACTERIA*

Bacterial species with GenBank accession numbers	**% Sequence similarity to *Calymmatobacterium granulomatis****
	%
Escherichia coli A14565	**91**
Klebsiella pneumoniae X87276	**95**
Serratia marcescens M59160	**91**
Enterobacter species U39556	**94**
Citrobacter freundii M59291	**91**
Salmonella typhimurium U90316	**91**
Hafnia alvei M59155	**91**
Erwinia amylovora X83265	**91**
Erwinia carotovora M59149	**91**
Yersinia enterocolitica Z49829	**90**
Yersinia aldovae X75277	**87**
Acyrthosiphon pisum symbionts M27040	**90**
Rhizobium leguminosarum X67233	**60**
Neisseria gonorrhoeae X07714	**69**
Caulobacter crescentas M83799	**58**
Bacillus subtilis X60646.	**58**

* values are based on the results of 1373 nucleotides

5.3.5.3 Phylogenetic analysis

Optimised sequence alignments were prepared using the 1373 bp of the 16S rRNA sequence of the bacteria from the monocyte co-culture (KH 22), frozen (KH 6) and formalin fixed paraffin wax embedded tissue biopsy specimens (KH 34) (excluding the primer sequences) and those of other organisms. These alignments were used to construct a phylogenetic tree according to the methods described.

Five of the six phylogenetic methods placed *Klebsiella* as the most closely related species with strong bootstrap values (Figures 78 to 83). All phylogenetic trees presented show the consistency of the three sequences forming a distinct group. The relationship of this distinct group shows the evidence of a new genus *Calymmatobacterium* within the taxon, *Enterobacteriaceae*. On the basis of the phylogenetic findings KH 22, KH 6 and KH 34 are members of a new genus, for which the name *Calymmatobacterium* is appropriate.

5.3.5.4 Sequence signatures

The sequence signatures as suggested by Woese (1987) for the alpha, beta and gamma subclasses of *Proteobacteria* form distinct groups. The sequence signatures of *C. granulomatis* (KH 22, KH 6 and KH 34) clustered within the gamma subclass of the *Proteobacteria*. The sequence signatures for this group are shown in table XVI. The RNA secondary structure prediction between positions 180 and 220 were based on the Zuker - Stiegler algorithm and are shown in figure 83. For purposes of comparison these RNA secondary structures for the alpha, beta and gamma subclasses are also shown.

5.3.5.5 GenBank nucleotide sequence accession numbers

The 16S rRNA sequences determined for *C. granulomatis* have been deposited in the Genome Sequence Database (GSDP) at Los Alamos National Laboratory (formerly GenBank) under the following accession numbers: *Calymmatobacterium granulomatis* monocyte co-culture - strain KH 22 - **AF010251**; *Calymmatobacterium granulomatis* frozen biopsy specimen - strain KH 6 - **AF010252**; *Calymmatobacterium granulomatis* from formalin fixed paraffin wax embedded biopsy specimen - strain KH 34 - **AF010253**.

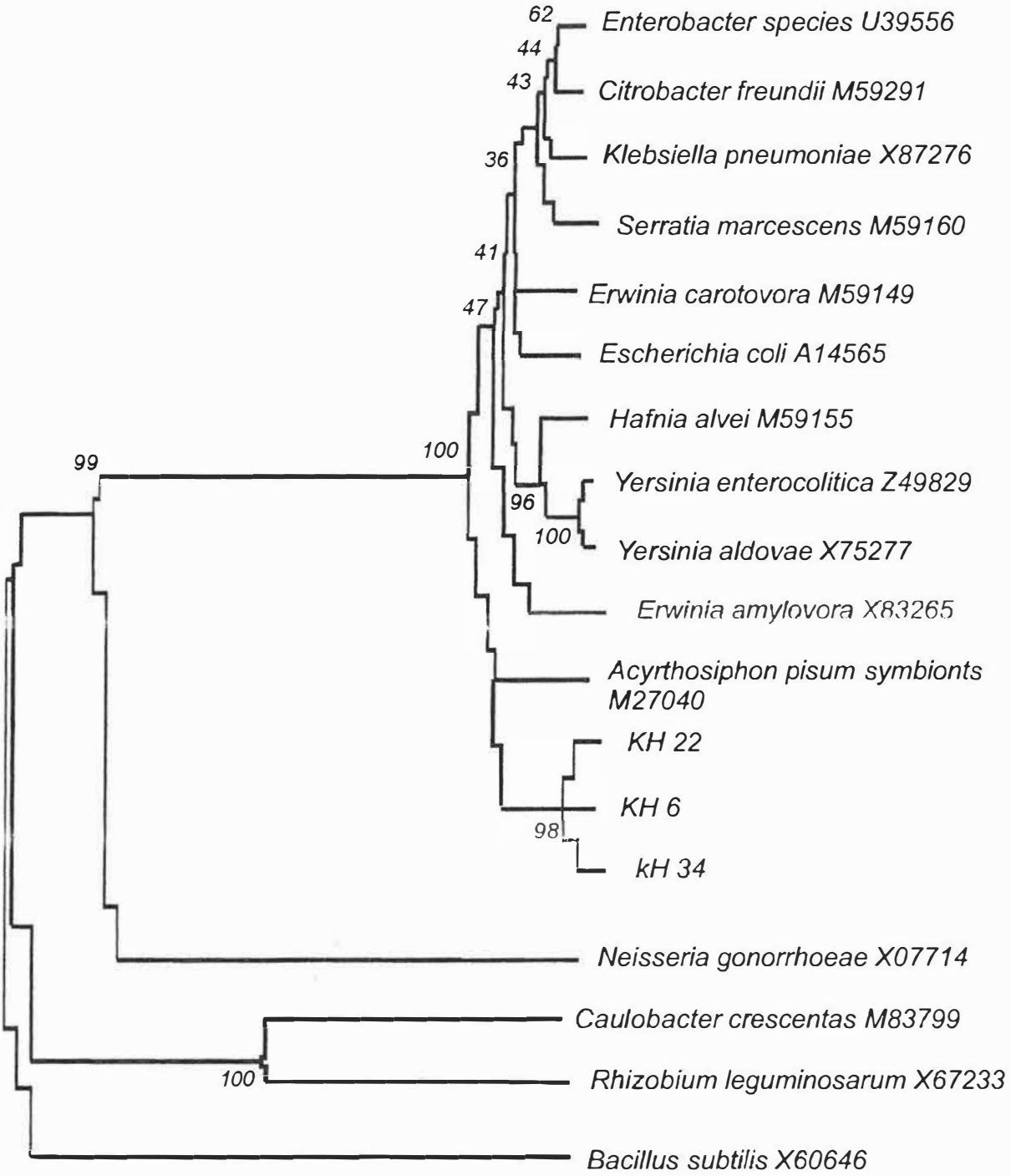


Figure 78: Dendogram showing the relationship of KH 22, KH 6 and KH 34 and other organisms. Clustering was performed by the unweighted pairwise grouping method of arithmetic average (UPGMA) from MEGA with 500 bootstrap. *Bacillus subtilis* was used as the designated outgroup. The tree is based on a comparison of 1373bp. Scale : each is approximately equal to the distance of 0,00159.

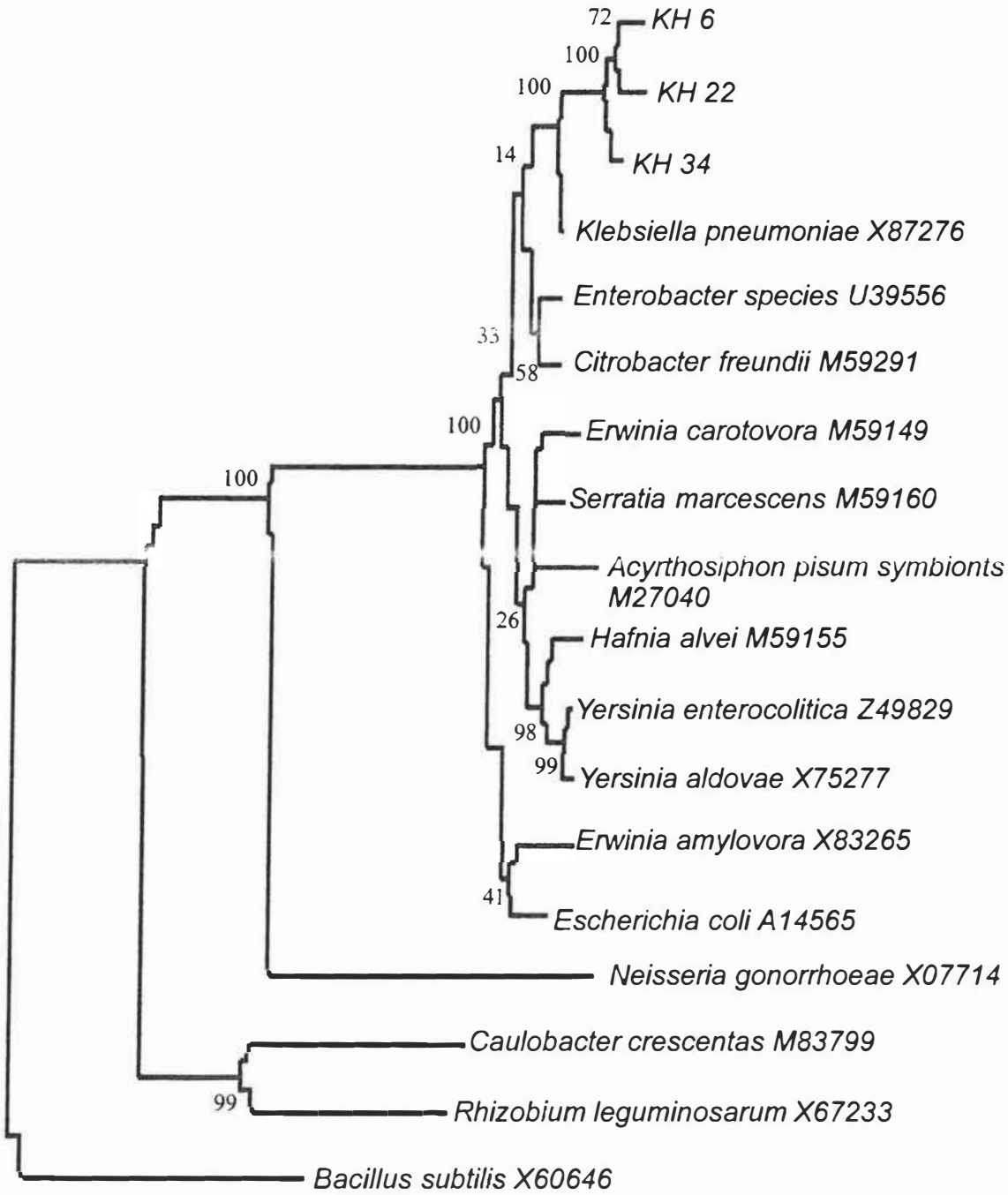


Figure 79: Phylogenetic neighbor-joining tree from MEGA using Tajima and Nei distances with 500 bootstraps. The tree is based on a comparison of 1373bp. Scale : each is approximately equal to the distance 0.0002573. The numbers above the branches are the percentages determined in a bootstrap analysis.

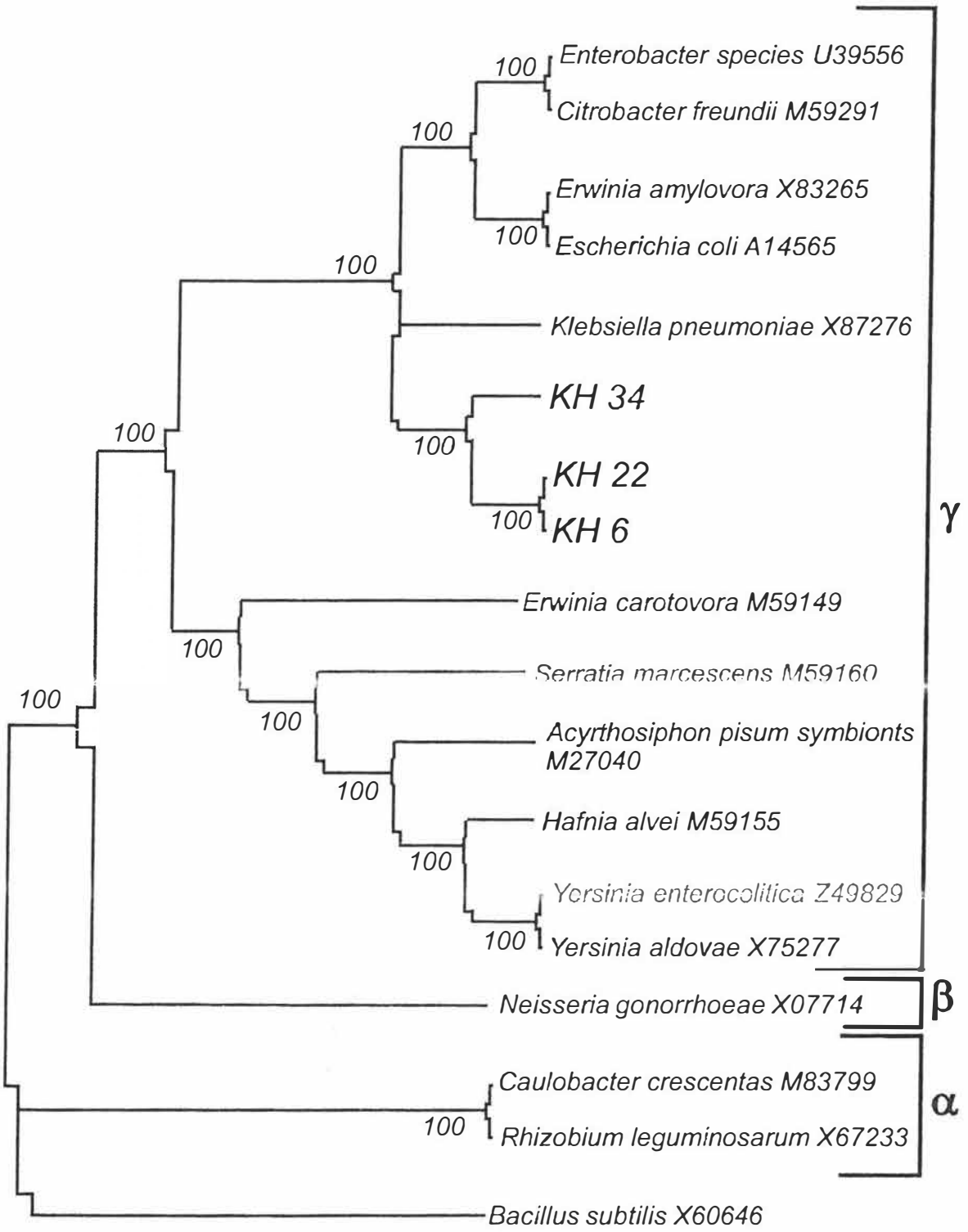


Figure 80: Phylogenetic positions of KH 22, KH 6 and KH 34 within the *Proteobacteria*. The tree was constructed using branch and bound maximum - parsimony from MEGA. The tree is based on a comparison of 1373bp. *Bacillus subtilis* was used as the designated outgroup.

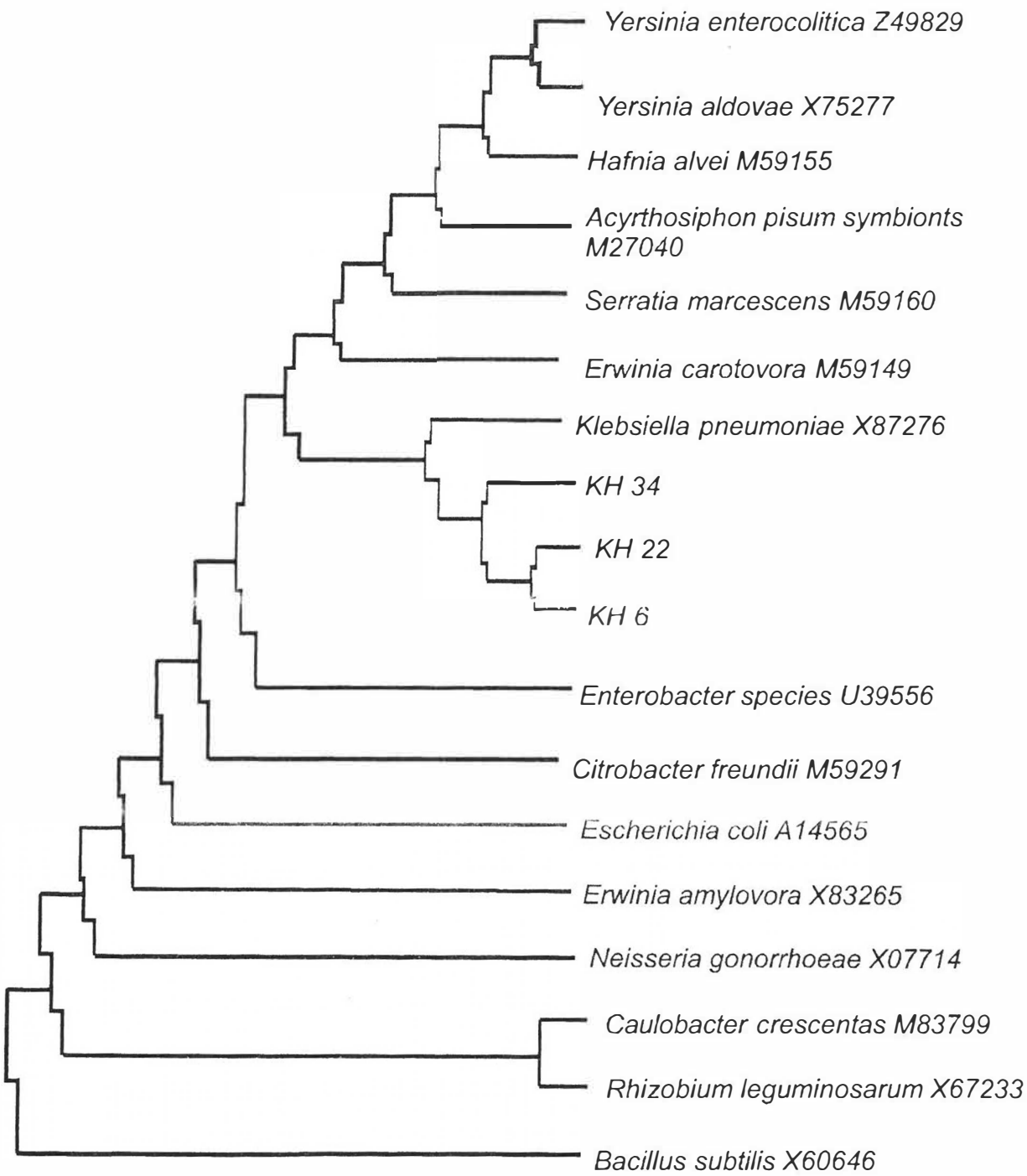


Figure 81: Heuristic maximum parsimony phylogram from MEGA obtained from the analysis of 16S rDNA sequences. The tree is based on a comparison of 1373bp. *Bacillus* was used as the designated outgroup.

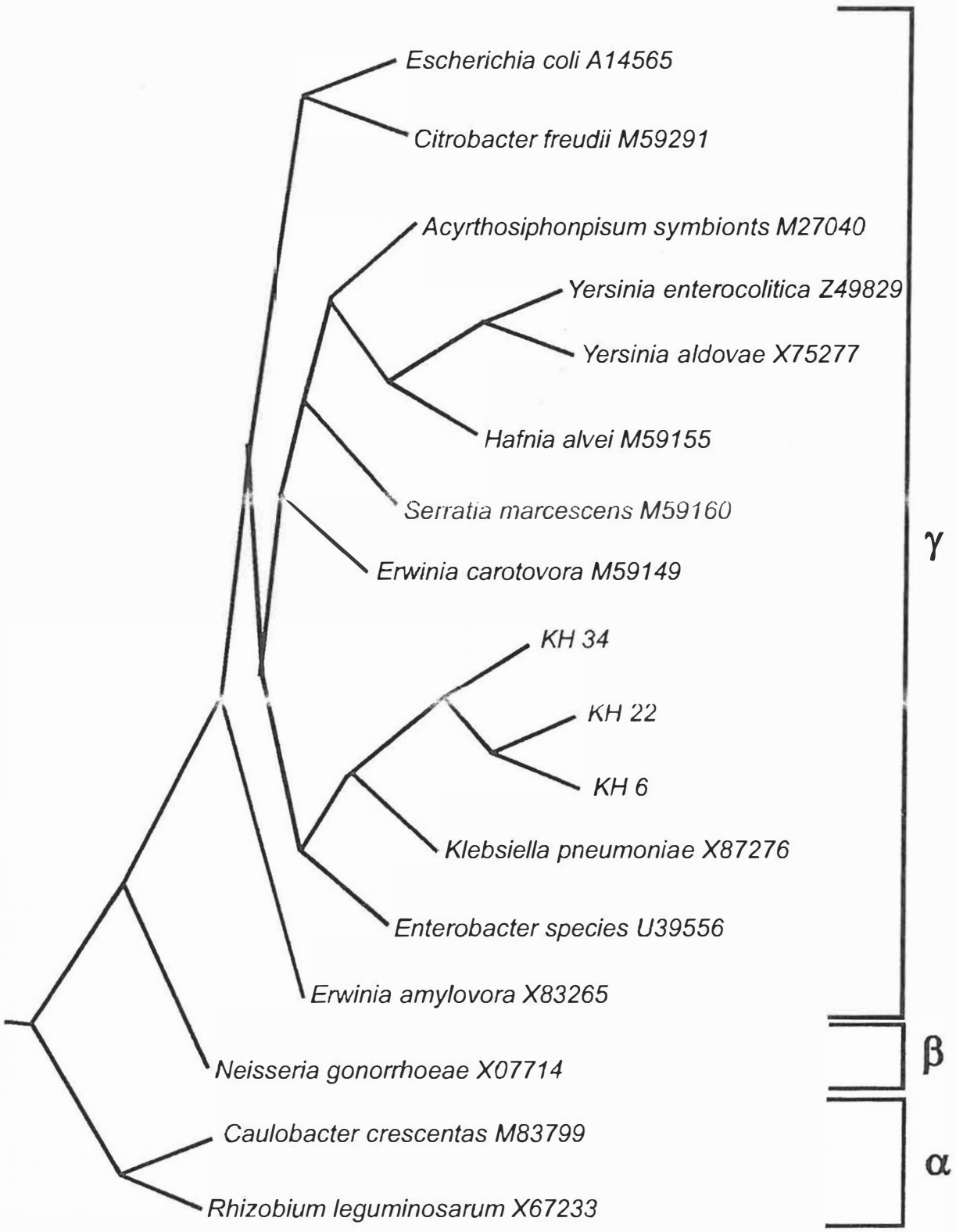


Figure 82: DNA penny phylogram from PHYLIP obtained from the analysis of 16S rDNA sequences. The tree is based on a comparison of 1373bp. *Bacillus* was used as the designated outgroup.

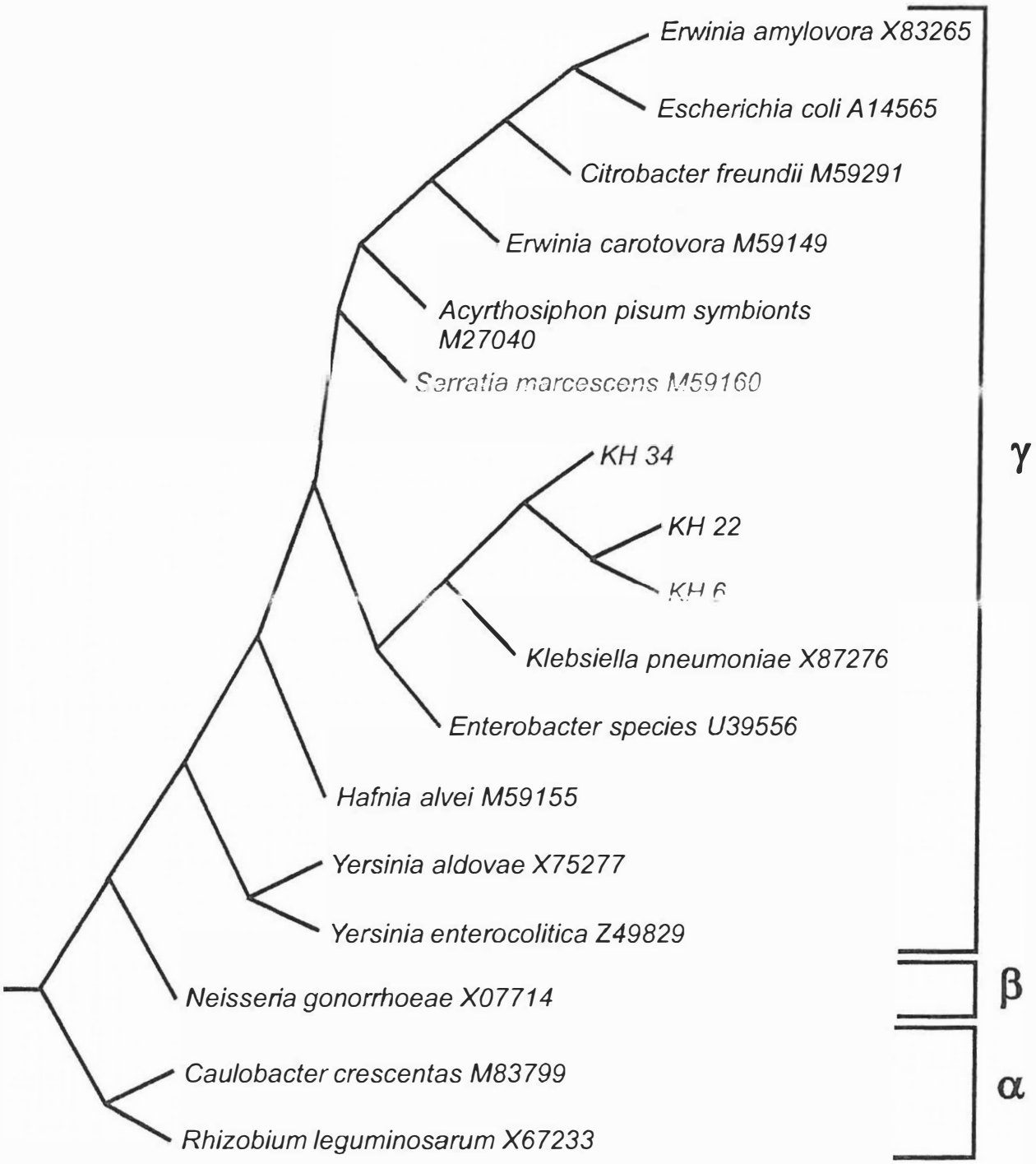
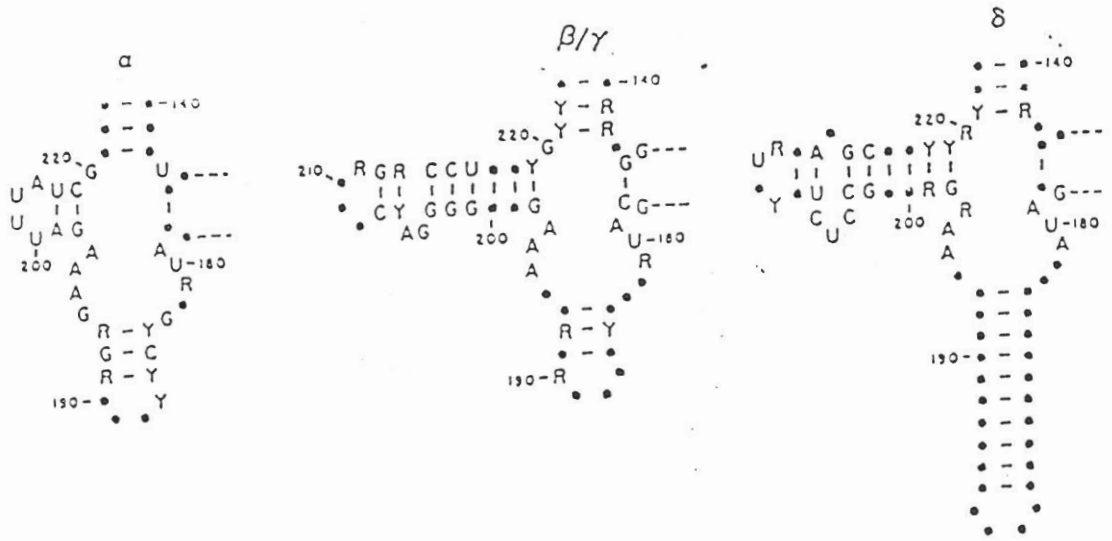


Figure 83: DNA parsimony phylogram from PHYLIP with 100 bootstraps obtained from the analysis of 16S rDNA sequences. The tree is based on the comparison of 1373bp. *Bacillus* was used as the designated outgroup.

TABLE XVI: SEQUENCE SIGNATURES FOR THE GENUS *CALYMMATOBACTERIUM*

Position*	**Nucleotide in #**			
	Proteobacteria			
	alpha subgroup	***Beta subgroup***	***Gamma subgroup* (&)**	**KH22, KH 6, KH 34 *Calymmatobacterium* ($)**
108	G	A	G	**G**
236	A	A	G	**G**
502	A	A	C	**C**
543	T	T	G	**G**
689	C	C	A	**A**
690	G	A	G	**G**
825	A	A	G	**G**
929	G	A	G	**G**
947	G	T	G	**G**

* *Escherichia coli* numbering (Brosius *et al*, 1978)
# Bases found in other members of the *Eubacteria.*
$ Bases found in (KH 22, KH 6 and KH 34) *C. granulomatis* species
& Bacterial species having the same base as *C. granulomatis*



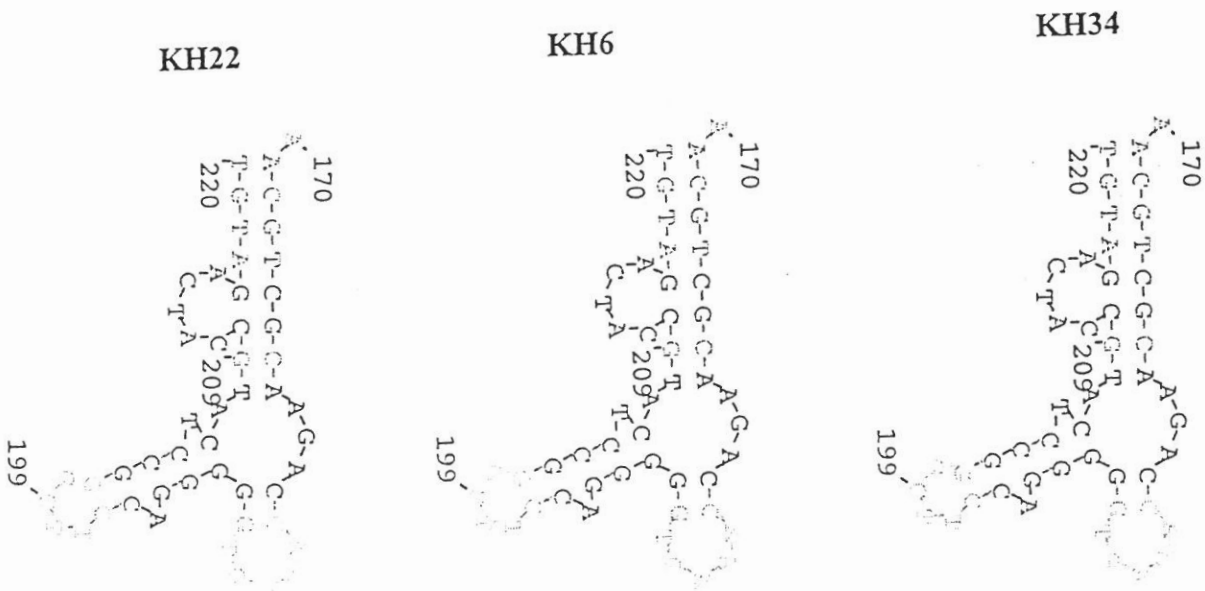


Figure 84: 16S rDNA secondary structures between nucleotides 180bp and 220bp of the three (KH6, KH22, KH34) derived 16S rDNA compared to those belonging to the alpha, beta, and gamma subdivisions of the *Proteobacteria* (Weisburg *et al* 1991).

5.4 DISCUSSION

The laboratory diagnosis of granuloma inguinale has been difficult, relying only on the observation of Donovan bodies in clinical specimens, which has a sensitivity of less than 70% (Richens, 1991). The absence of any serological tests and the inability to cultivate *Calymmatobacterium granulomatis in vitro* has hampered the identification and characteristion of the organism.

In - vitro amplification technology has become a powerful tool for fast and precise production of DNA fragments or even entire genes. Although the PCR has been known to allow the isolation of entire genes, the nucleotide sequence determination requires cloning procedures. Sequencing of the cloned amplified DNA may result in false nucleotide determination due to misincorporation by the *Taq* polymerase during amplification reaction (Saiki *et al*, 1988). These misincorporations can be resolved by sequencing several clones (Weisburg *et al*, 1991) or to directly sequence the amplified product in both direction (Edwards *et al*, 1989). This is the first report whereby the 16S rDNA of *C. granulomatis,* a previously uncultured organism, implicated in the aetiology of granuloma inguinale was amplified, cloned and sequenced from monocyte co-cultures, frozen and formalin fixed paraffin wax embedded tissue biopsy specimens. The sequencing strategy resulted in the determination of 1370 nucleotides for each gene product, representing approximately 90% of the total primary rRNA structure.

In preliminary experiments of smear positive cultures of tissue specimens no amplification was achieved, although RapiDiff stained smears were positive for Donovan bodies. This may have been due to the specimens being stored inappropriately for a considerable period of time, hence the quality of DNA as well as the yield could have been affected due to residual fixation, chemicals and tissue debris (Kallio *et al*, 1991). In subsequent experiments amplification of bacterial DNA was successful from the majority of specimens since the inhibitory effects of fixatives and / or proteins were overcome by further purification of the tissue extracts by the phenol / chloroform extraction method (Sambrook *et al*, 1989). In addition, since there are many potential sources of PCR contamination (Kwok and Higuchi, 1989; Schmidt and Relman, 1994), additional control measures were

adopted as recommended in the proposed guidelines for molecular diagnostic methods (Enns *et al*, 1994). This was unidirectional workflow and physical separation of reagent preparation, sample preparation, amplification and product detection. Each laboratory was equipped with dedicated equipment, and good standard laboratory practices enforced. Negative controls used for amplification were water in place of target DNA and reagent blanks containing all of the reaction components without the addition of template. During sample preparation, tubes containing sterile distilled water were processed with each batch of samples used ie. culture and tissue biopsy specimens. Furthermore, amplification was performed for each batch of samples / specimens at separate times, over a one year period in order to prevent cross contamination between specimens.

A rapid system for the cloning of unmodified PCR products has been described previously (Marchuk *et al*, 1990). In this study the use of T:A cloning system of unmodified PCR products into p MOS*blue* T - vector was successful. This facilitated the generation of double stranded DNA templates from the recombinant clones which were suitable for sequence analysis.

Assays based on nucleic acid detection have the potential for greater sensitivity than immunological methods. In this study the use of oligonucleotide probes which have been designed for the detection and identification of bacterial pathogens in normal sterile body fluids, were used in the hybridisation of the restricted recombinant clones from the monocyte co-culture, frozen and paraffin wax embedded biopsy specimens. Hybridisation experiments performed using the specific universal bacterial (RDR245) and Gram negative (DLO4) oligonucleotide probes suggested that the restricted fragments from the recombinant clones from all three sources which had also been sequenced originated from Eubacteria. Conclusive evidence that the organisms were Gram negative and not Gram positive was the fact that no hybridisation signal was obtained with the Gram positive probe (RWO3). The Gram positive probe (RWO3) corresponds to two sequence signatures identified in the 16S rRNA (Greisen *et al*, 1994), the one being a C residue located at position 1207 and an A residue located at position 1198 of the *E. coli* 16S rRNA gene. The residues at these positions are G and T for positions 1207 and 1198 respectively which further confirms the sequence as that from a Gram negative organism. It could be argued that the 30bp

vector sequence included in the restricted fragment from the recombinant clones may have cross reacted with the respective probes upon hybridisation. However, the high temperature during hybridisation (68°C) and the high stringency washes at high temperature (0,1 x SSC; 68°C) therefore argue against this. Oligonucleotide hybridisation of *C. granulomatis* probes can be applied and used for the *in situ* detection and identification of individual species, as well as phylogenetic groups of cultured and uncultured organisms from which a sequence has been retrieved. This aspect of the work is ongoing.

The precise method for assessing levels of relatedness of different taxa is by determining the nucleotide sequences. DNA sequence analysis of the entire genome is clearly impractical. On the other hand, it is practical to determine the nucleotide sequence that codes for a region which appears to encompass the desired degree of conservation for the purpose at hand. The principle of using rRNA sequences to characterise micro-organisms has gained wide acceptance. The analysis of nucleic acid sequences coding for 16S rRNA is particularly useful for phylogenetic analysis which reflect evolutionary pathways (Lane *et al*, 1985) and for characterisation of organisms of uncertain affiliation (Relman *et al*, 1990). Furthermore, the variable domains of the 16S rRNA molecule show considerable sequence diversity even between closely related species and therefore are useful as target sites for the construction of specific probes for the rapid identification of micro-organisms (Wilson *et al*; 1988). For any meaningful analysis, a major part of the 16S rRNA gene of at least 1000bp needs to be sequenced (Murray *et al*, 1990). Comparative sequence analysis will reveal whether it is novel sequence originating from an unknown organism. In this study, 1380bp of the 16S rDNA was sequenced, therefore allowing for the outcome of the sequence analysis to be taken with a degree of confidence.

The availability of resources and technology for rapidly determining nucleotide sequences in selected regions of 16S rRNAs has led to some attempts to base phylogenetic schemes on levels of 16S rRNA homology alone. Whilst 16S rRNA sequencing methods yield excellent data and provide insights into evolutionary relationships at the highest taxanomic level in bacteria, it is unclear how these methods can be applied to resolve relatedness or distinguish subspecies or strains of the same species. This is in part related to the fact that a limited conserved region of the entire genome is reflected by the 16S rRNA genes and

the fact that the depth of branching reflects in part the age of a particular branch; interspecies depth of branching may differ from genus to genus and even within a genus (Stackebrandt and Goebel, 1994). Additional resolving power can be ontained by adding restriction fragment length polymorphism technology.

In this study the overall level of homology for the almost complete analysed 16S rDNA sequences was >99% for the three sequences coming from different sources which suggests and supports that the nucleotide sequence as obtained from monocyte co-culture and biopsy specimens were from the same bacterial species. Although there was base pair variation between the three sequences this would suggest some diversity within the disease causing species. There was less than 2% difference in the sequence analysis from the three different sources and this sequence variability within a species has not been properly defined. The high level of homology amongst the three 16S rDNA sequences from patients with microscopically proven granuloma inguinale suggest that the organism could be identified as a member of a particular species, and the definition of generic relationship provided evidence that granuloma inguinale is caused by this particular species.

It is generally accepted that strains with 16S rDNA homology values of less than 97,5% are unlikely to belong to a single species (Stackenbrandt and Goebel, 1994). On the other hand identical or nearly identical sequences are not proof of identity at the species level (Eckloff *et al*, 1994). The best solution to this dilemma would be to grow and isolate bacteria and perform the quantitative DNA-DNA hybridisation which is the standard method for the designation of species. However, for non-cultivable bacteria, or cultivable only at very low efficiency, amplification and sequencing of rDNA is the method of choice for laboratory use. Phylogenetic relationships have been assessed at all taxonomic levels by analysis of rRNA and/or of the gene that encodes it. Current analyses involve the application of DNA-rRNA hybridisation, 5S, 16S and 23S rRNA nucleotide sequencing. Data obtained from these methods provide the best basis at the present time for determining phylogenetic relationships among all bacteria (Murray *et al*, 1990).

The utility and validity of bacterial phylogeny and identification are increasing on the basis of ribosomal sequence information. Therefore, it is important to know to what degree the

extensive polymorphism observed in genomic sequences of many bacteria extend to 16S rDNA sequences. There are no universally acceptable criteria for deciding how many base deletions and / or substitutions in the 16Sr DNA sequences are sufficient to justify establishment of a new species. The use of sequence data is assessed on the comparison with a type strain by pairwise comparisons, or by comparison with a consensus sequence, though these approaches have their merits, comparison with a consensus sequence minimises disparity and avoids distortion due to selection of an arbitrary standard and it is unclear as to which is the ancestral sequence (Eckloff *et al,* 1994). The sequences derived from the three samples of patients with microscopically proven granuloma inguinale have been submitted to the GenBank database. There was greater than 99% sequence homology between them and therefore this sequence data would be available for comparative analysis of *C. granulomatis* strains.

Little is known about intraspecies variations of 16Sr DNA sequences. Such diversity of sequences for one species may be caused by acquired genotypic changes such as those leading antibiotic resistance, species heterogeneity, heterogeneity of the 16S rRNA copies (Relman *et al*, 1991) within a strain or sequencing errors. The 16S rDNA sequences of *C. granulomatis* appear to be relatively stable, but due to the small sample size in this study no conclusions could be drawn. There are a number of possibilities for the observed sequence differences. There is evidence that little diversity of the 16Sr DNA sequences of *Francisella* (Forsman *et al*, 1994) and *Legionella pneumophilia* (Fry *et al*, 1989) occurs, whilst for *Borrelia burgdoferi* strains, up to 1,0% divergence has been demonstrated (Marconi and Garon, 1992). Eckloff *et al*, (1994) compared the 16S rDNA sequences from five isolates of *Helicobacter pylori* finding deviations from the consensus sequence at 13 separate positions which could be attributed to clonal origins. Relman *et al*, (1990) reported the unique 16S rRNA gene sequences from tissues of three unrelated patients with bacillary angiomatosis, whilst a sequence obtained from the tissue of the fourth patient differed from the sequence found in the other three patients at four of 241 base positions. It is suggested that these sequences are all related to the 16S rRNA sequence of the same organism, but sequence heterogeneity could indicate the presence of related strains within diseased tissue. Similarly a small number of nucleotide differences are known to exist within the several copies of the *E. coli* rRNA gene. Another possibility for the observed

sequence difference is the fidelity of the enzyme, *Taq* polymerase. *Taq* polymerase could misincorporate nucleotides, particularly during the amplification of damaged DNA which would be observed after direct sequencing of PCR products. This emphasises the importance of cloning and sequence analyses of multiple clones of the amplified products from the same and different specimens in order to obtain a "near - perfect" sequence (Weisburg *et al*, 1991). It would be interesting to consider whether the few differences observed in the 16S rDNA sequences of *Calymmatobacterium* strains are reflected in phenotypic traits.

The 16S rDNA sequencing method in this study was able to identify bacteria from all three sources accurately as belonging to the gamma subclass of *Proteobacteria*: the genus being unique. The sequence analysis is easy to interpret when a newly characterised sequence can be compared to a known sequence available in the database. However, presently none of the obtained sequences could be compared to any known sequences of *C. granulomatis* from the available databases as these are not known. When compared to other sequences within the alpha, beta and gamma groups of *Proteobacteria*, the phylogenetic treeing analysis confirmed that *C. granulomatis* belongs to a separate branch of the gamma subclass of the *Proteobacteria* which is characterised by species belonging to the genera (Woese *et al*, 1990). The group *Proteobacteria* contains some 200 genera encompassing a large proportion of Gram negative bacteria, which can be divided into at least five subclasses. The outstanding attribute of the major phylogenetic branches (alpha, beta, gamma, delta and as yet an unnamed subclass) within the purple bacteria and their relatives is the diversity of shape and physiology and the term *Proteobacteria* at the class level forms a major and diverse assemblage consisting of a related set of lineages (Stackenbrandt *et al*, 1988).

Whilst the causative agent has been proposed to be morphologically related to *Klebsiella* group of bacteria, although phylogenetically related to some extent, it is clearly distinct. The phenotypic characteristics of the members of the Genus *Klebsiella* do not resemble those of *Calymmatobacterium* Genus. Currently, there are no phenotypic characteristics which could easily and readily be used by clinical laboratories to differentiate *Calymmatobacterium* from other members of the *Enterobacteriaceae*, particularly

Klebsiella. In addition there are no conventional or commercial tests which can reliably identify and differentiate these organisms. The use of PCR with specific primers targeting the 16S rRNA gene should accurately identify and differentiate strains of *C. granulomatis* from other members of the *Enterobacteriaceae*. This method would be a valuable and efficient technique which could be used by clinical microbiologists. Alternatively, the specific sequences could be used for the development of a probe for use in DNA immunoassays.

The members of *Enterobacteriaceae* genera exhibit fundamental differences in biochemical and physiological properties. Many species within the *Enterobacteriaceae* such as *Klebsiella, Serratia, Citrobacter, Salmonella, Escherichia* can easily and readily be cultivated on cell free media which differs from *Calymmatobacteria* in being a fastidious organism which does not grow on cell free medium. This clearly indicates the specific physiologic requirements of the different species. Although the sequences of *C. granulomatis* (KH 22, KH 6 and KH 34) are closely related to *Klebsiella* they form a distinct group within the taxon *Enterobacteriaceae* and it would be appropriate to retain the genus *Calymmatobacterium* which is presently supported by the differential phenotypic characteristics of the two groups. Similarly, Forsman *et al* (1994) in their sequence analysis of *Francisella* strains observed unusually high levels of sequence homology between the 16S rRNA sequences of *Wolbachia persica* and *Francisella* strains and suggested that this does not necessarily mean that *W. persica* should be placed within the genus *Francisella*, but rather does indicate that the species should be further characterised phenotypically to determine its relationship to *Francisella* species.

Bastian and Bowden (1996) have targeted the *phoE* gene, which encodes for a porin protein. This outer membrane protein consists of conserved membrane - spanning segments and hypervariable surface exposed regions within the members of the family *Enterobacteriaceae*. The amplification of a *Klebsiella* - like sequence from the genital lesions of patients with granuloma inguinale could be used for diagnostic purposes, whilst the use of this region for taxonomic purposes needs to be further investigated. A thorough revision of the generic and species classification of this group needs to be pursued, since, 16S rRNA sequences can be used routinely to distinguish and establish relationships

between genera and well - resolved species, whilst very recently diverged species may not easily be recognisable (Fox *et al*, 1992).

Today's improved technology will allow the design of probes complimentary to specific regions and can form the basis of more rapid and sensitive means of detecting bacteria in clinical samples as compared to the traditional methods of *in vitro* cultivation and identification. The ability to cultivate *C. granulomatis* in tissue culture and the potential to cultivate the organism on artificial cell free media will allow phenotypic characterisation and fulfillment of Koch's postulates, complimenting the genotypic characterisation.

CHAPTER 6.0
OVERALL DISCUSSION

Granuloma inguinale caused by *Calymmatobacterium granulomatis* is considered to be one of the rarer causes of genital ulcers. It remains a significant health risk, particularly in certain developing countries. The use of antimicrobial agents may have reduced the prevalence of granuloma inguinale in many parts of the world and therefore, in most developed countries, the incidence of granuloma inguinale declined concurrently with other sexually transmitted diseases. In western countries such as the United States, United Kingdom, Europe and Canada, granuloma inguinale is non existent, whilst the disease still exists in specific geographical regions and has emerged as a significant cause of genital ulcer disease in the Kwa Zulu / Natal region of South Africa. The prevalence of granuloma inguinale in the Kwa Zulu / Natal region has steadily increased since the 1980's. Morbidity due to this disease in relation to the HIV epidemic emphasises the need for improved control measures (O' Farrell, 1991).

Widespread diagnostic screening, followed by treatment is urgently needed to eradicate the disease. This becomes difficult because of the relatively low infectivity, long incubation periods and the lack of an animal reservoir, however, it makes granuloma inguinale a potential candidate for control by immunisation. Chapter 2 of this thesis deals with the epidemiology, aetiology, clinical features and laboratory diagnosis. The origin, mode of transmission, progression of disease, the problems encountered in the clinical and laboratory diagnosis of granuloma inguinale have been delineated.

The clinical features of granuloma inguinale generally correspond to the earlier descriptions with lesions varying from small to moderate to florid and extensive lesions. The latter results in elephantiasis, mutilation of the genitalia with residual scarring.

The fastidious nature and the inability to grow the organism *in vitro*, has been the main reason why there are still many unanswered questions about the pathogenicity and virulence factors of the organism and the epidemiology of the disease. On the basis of microscopic analysis of the morphology it has been impossible to recognise and distinguish any

subspecies. In addition there are no available serological tests for the alternate diagnosis of the disease. The appropriate diagnosis of granuloma inguinale is important for public health reasons. Granuloma inguinale by virtue of causing painless ulcerative lesions which bleeds easily on contact increases the risks of acquisition and transmission of HIV through sexual contact. This is well known with genital ulcerative conditions (Wasserheit, 1992; Clottey and Dallabetta, 1993).

Presently, the diagnosis is based only on the microscopic visualisation of Donovan bodies in tissue smears or biopsy specimens, which has limitations, since microscopy tends to be insensitive. For this reason it is important to have a laboratory tool, such as the cultivation of the organism for the appropriate diagnosis of granuloma inguinale. The aims of this study were several: to develop and establish an *in vitro* method for the cultivation of *C. granulomatis*; to analyse the ultrastructural morphology of the organism in culture and in direct tissue biopsy specimens and finally to identify the organism from the monocyte co-culture and in tissue biopsy specimens using molecular methods based on the 16S rDNA sequence analysis.

In this study the culture technique was established using peripheral blood mononuclear cells (PBMNC) in a monocyte co-culture system to cultivate *C. granulomatis*. This technique initially made use of tissue biopsy specimens and later tissue scrapings where the number of bacteria in the specimens varied in concentration. These studies showed that the culture technique could be used for the recovery of organisms from a considerably small initial inoculum. The fact that the recovered bacteria morphologically resembled the described Donovan bodies and fluoresced with immune sera suggested that this technique was highly sensitive and specific and therefore has the potential to be used for the diagnosis of granuloma inguinale.

It is surprising that most of the earlier studies relied on the use of fertile chick eggs or cell free media using fertile chick egg yolk as components. Earlier researchers have reported the growth of what they believed to be *C. granulomatis* on cell free media (Anderson *et al*, 1943; Dulaney *et al*, 1948; Goldberg, 1959; Goldberg, 1962). However, in this study no growth was obtained using this extensive list of media, despite following the published

methodology for the preparation. It is possible that unpublished details or unidentified differences in the ingredients may be the reason for these negative results. Another explanation could be that previously reported isolates of *C. granulomatis* which grew on cell free media belonged to a different species. This is the first report on the use of monocytes as a specialised co-culture system for the extensive propagation of the organism. The choice of peripheral blood monocytes for culture was based on the visualisation of the causative agent within monocytes of specimens (smears and biopsy) of infected tissue. Such a system allows human monocytes and macrophages to control the growth of microbial pathogens and provides an opportunity to study host - parasite interactions (Carvalho de Sousa and Rastogi, 1992)

The optimisation of the culture method using pooled PBMNC from HIV negative blood donors together with autologous or foetal calf serum and the treatment of genital biopsy specimens with amikacin, vancomycin and metronidazole proved to be successful.

Using light microscopy, the bacteria in the monocyte co-cultures were similar in morphology to those seen in direct smears. These appeared as either definite short coccobacillary or pleomorphic encapsulated forms. In early culture reports *C. granulomatis* had been described as being pleomorphic bacteria (Anderson *et al*, 1943; Rake and Oskay, 1948; Goldberg, 1962). In this study the pleomorphic nature of the bacteria was confirmed. However, the morphology varied in the primary and secondary cultures. Two forms were evident in the primary and secondary cultures, with the long bacillary forms predominating in primary cultures and the coccobacillary forms in subsequent cultures suggesting that these organisms were in a process of bacterial elongation, cell division and multiplication. During subsequent passages the capsule and the single or bipolar staining reaction of the organisms were still evident. This morphological appearance is attributable to viable cultures demonstrating the varying stages of growth suggestive of an optimal growth environment. The observation of the long tapered bacterial cells undergoing cell division in the early cultures and the presence of the coccobacillary forms, could suggest the presence of two morphological two forms of *C. granulomatis* and this variation was evident during the different stages of the growth being the logarithmic (long tapered forms) and stationary stages (coccobacillary forms). These stages in other Gram negative bacteria have

been recognised and are known to differ in ultrastructure and function (Falklow *et al*, 1992) such as in their ability to attach to the cells or extracellular matrix of the host (Finlay *et al*, 1989) and to enter the host cell (Lee and Falklow, 1990). Therefore the pathogenic mechanisms of adherence and invasion of *C. granulomatis* could be related to the two forms of bacteria described.

Anderson *et al* (1945), reported the presence of *C. granulomatis* specific antibodies in convalescent sera of patients with granuloma inguinale, whilst others have found similar responses when using *Klebsiella* antigen instead of *Calymmatobacterium* antigen (Rake, 1948; Packer and Goldberg, 1950). Although there are controversies regarding cross reactivity with *Klebsiella* species in such a test, we did not observe this with the three species of *Klebsiella* used. These results confirm the lack of cross reactivity with other bacterial antigens. All the monocyte co-culture isolates of the study specimens and the direct smears containing Donovan bodies which were used as controls showed bright fluorescence with immune sera.

Infection of the mononuclear cells with *C. granulomatis* did not cause any significant cytopathology. The cytoplasm of the infected host cell was occasionally vacuolated but not necrotic. Intracellular bacteria can produce toxicity and lysis of host cells, particularly during the extended incubation times. To overcome this the infected cells were examined at 48 hours which decreased the length of incubation. Extracellular and intracellular growth, acidification of the medium and production of toxins could also influence the viability and attachment of the monolayers. The relative viability and integrity of the monolayers were evaluated microscopically by direct microscopic observation.

A method has been developed for the culture of *C. granulomatis* which can be applied to routine clinical specimens like ulcer scrapings as well as biopsy specimens of large lesions. The successful culture of the aetiological agent of granuloma inguinale will enable us to collect a sufficient number of bacterial strains to study the biological characteristics and virulence attributes of this organism, to determine its antimicrobial susceptibility profile and to develop a definitive diagnostic test. This would lead to a better understanding of the pathogenesis of this disease.

Transmission electron microscopy confirmed the ultrastructural morphology of the organism in the monocyte co-culture specimens. *C. granulomatis* has a characteristic Gram negative cell wall structure with a well defined electron dense capsule. The bacteria were viable in monocyte co-culture, as shown by the presence of extracellular dividing bacteria.

The appearance of the organisms in tissue biopsy specimens was also typical of Gram negative bacteria having the same morphological features as those seen in the monocyte co-culture specimens. However, there were distinct variations in the widths and densities of the capsules. In the latter the capsules were well defined, with a uniform width, whilst the bacteria in the biopsy specimens had capsules with varying widths. The size of capsule of the bacteria from the monocyte co-culture was increased, whilst in tissues the capsule varied considerably. This altered morphology of the capsules of *C. granulomatis* may have been influenced by the environmental growth conditions, especially when incubated in an enriched medium *in vitro*.

The capsular material is widespread among pathogenic bacteria and the type and rate of synthesis may have a bearing on the pathogenicity of the organism. The capsule is particularly important in bacteria whose strategy for survival in the host depends on evasion of phagocytosis or serum resistant characteristics. Protection is thought to be a function of the physical properties of the capsule, including hydrophobicity, charge and viscosity. The bacterial growth environment often determines the presence, size and composition of the capsule. Bacteria isolated from host tissues, incubated with host serous fluids, or grown in diffusion chambers *in vivo* are often covered by a continuous layer which is absent from isolates subcultured *in vitro* (Brogden and Clarke, 1997).

Fibrillar material was frequently present within the vacuole of the macrophages within which the organisms were present. It has been suggested that the fibrillar material represents degrading bacteria and is antigenic and can be confirmed by immunogold labelling as has been shown with *Erlichia* infected cells (Popov *et al*, 1995). In this study the fibrillar material were extensions of the capsule, extending in some cases to the internal membrane of the vacuole and these may well be the products of degraded bacteria, though the internal components of the bacteria were well defined and suggestive of intact cellular

components. The fibrillar material could be *Calymmatobacterium* antigen and needs to be isolated and characterised by an immunogold labelling method .

In the biopsy specimens bacteria were seen and multiplied both intra and intercellularly and heavily infected cells were more often observed to be necrotic. These observations suggest that *C. granulomatis* multiplies both intra and extracellularly and the cells with intracellular bacteria develop pathological alterations and the bacteria are at times destroyed within the vacuole. The pathogenic mechanism by which *C. granulomatis* are killed needs to be further investigated. Although the histopathological appearance of granuloma inguinale is specific, the absence or low numbers of Donovan bodies following treatment and the variable histopathological manifestations for other sexually transmitted diseases make it difficult to differentiate granuloma inguinale on histological grounds alone.

In an effort to determine the identity and characteristics of *C. granulomatis*, 16S rDNA from the infected monocyte culture, frozen and formalin fixed paraffin wax embedded biopsy specimens was amplified with broad range primers, cloned and sequenced.

Sequence based identification of new pathogens, utilises nucleic acid amplification and *in situ* oligonucleotide hybridisation to detect micro-organisms thought to be associated with a disease process. These techniques have been useful in studying organisms which have not been characterised because they have proven, as yet to be uncultivable. The use of broadly conserved bacterial 16S rRNA sequences as targets for gene amplification has been a method of choice. The amplified products are sequenced and their phylogenetic relationship to 16S rRNA of other bacteria determined. The establishment of similarity with a known species allows the subsequent rationale for the development of tests to aid in the characterisation of the organism and confirm disease associations. The major problem in clinical microbiology is to associate a micro-organism with a disease. Microscopically the presence of Donovan bodies is pathognomonic of *C. granulomatis* in tissue smears of patients with granuloma inguinale and the utility of such samples for 16S rDNA analysis found that the three sequences obtained from three different sources had a homology of 99% and all three belonged to a unique genus within the gamma subgroup of *Proteobacteria*. This confirms the association of the aetiological agent with the disease,

granuloma inguinale and *Calymmatobacterium,* being the unique genus.

The control of granuloma inguinale is dependent on an effective primary health care programme and the availability of inexpensive and rapid diagnostic tests. Although often characteristic, the lesions of granuloma inguinale can mimic clinical manifestations of other diseases. The diagnosis of granuloma inguinale largely depends on the visualisation of the organism in tissue smears or histologically in biopsy specimens. This method lacks sensitivity and the presence and true nature of Donovan bodies cannot be confirmed by any other test. The effect of antimicrobial treatment of infected persons can be monitored by the absence of the organism in subsequent smears. Improvements to existing methods by developing specific antibody assays or bacterial DNA analysis techniques would contribute to the optimal diagnosis, effective treatment and control of granuloma inguinale.

The detection of *C. granulomatis* in clinical specimens has been essential for the diagnosis of granuloma inguinale. The limitations of this method is the requirement for the presence of at least 10^4 organisms per gram of tissue and hence lacks sensitivity. In recent years the PCR technology has proven to be valuable for the direct demonstration of fastidious pathogenic micro-organisms in clinical material. DNA amplification techniques have many advantages over conventional methods, they are rapid, sensitive, specific and is of particular value when other methods to detect micro-organisms have limitations. This may occur when micro-organisms are non-cultivable *in vitro* or in the case of infections with undefined pathogens. In cases where the nature of the causative micro-organism is unknown, broad range primers, which are specific for prokaryotic DNA, could be used to amplify ribosomal DNA and by sequence analysis of the amplified DNA a rapid preliminary diagnosis of the infectious agent can be obtained (Relman *et al*, 1990; Ward *et al*, 1990; Weisburg *et al*, 1991). PCR will allow the detection of only a few bacteria in lesion exudate, and hence it has great potential in the diagnosis of granuloma inguinale. Certain control measures have to be followed as previously mentioned in order to ensure optimal results (Kwok and Higuchi, 1989). The distinction between viable and non-viable organisms has to be carefully monitored as well as certain inhibitors in the clinical specimens might render a false negative result. Sequence analysis would enable one to design specific PCR primers or probes for use in amplification reactions or *in situ* PCR

hybridisation for the identification of *C. granulomatis* in clinical specimens.

Eradication of granuloma inguinale is possible because treatment of severe infections with an aminoglycoside and erythromycin has been shown to be highly effective (Hoosen *et al*, 1996) with little or no evidence of dissemination of the disease. In addition there are no indications of development of resistance to these antimicrobial agents. As with many infectious diseases the future prospects for a *C. granulomatis* vaccine needs to be considered for the prevention of acquisition and development of granuloma inguinale.

In the early 1950's granuloma inguinale was prevalent in most parts of the world, but slowly disappeared with few areas remaining as specific foci. Due to this eradication from most developed countries one could hope for the world wide eradication of granuloma inguinale in the future.

Due to the inability to cultivate *C. granulomatis in vitro* it has been impossible to isolate, purify and test individual antigen/s components for this pathogen. This problem could now be overcome by using the established monocyte co-culture technique for the preparation of antigenic components or could be circumvented by using recombinant DNA for the expression of *C. granulomatis* antigens. In South Africa, like in any other developing country it would seem relevant that there should be priority for the development and use of vaccines for sexually transmitted diseases, namely chancroid, chlamydia, syphilis and granuloma inguinale for the control of these diseases, since the high prevalence of these diseases in many developing countries is a risk factor for the spread of HIV. Basic research aimed at vaccine development, as well as applied research on safe, affordable and effective therapy regimens are needed.

It may not be possible to develop a vaccine that would induce sterile immunity. However, a vaccine that would supply only protection against symptomatic disease may be effective enough to eradicate granuloma inguinale. This would not prevent the recipient from acquiring the organism and contracting the disease, but would prevent the development of lesions and thus stop the transmission of the disease.

The use of the culture methods as described in chapter 3.0 would further enable the *in vitro* study of *C. granulomatis* micro-organism, the use of electron microscopy would enable the correlation of the organelles involved in the pathogenic processes and the sequence analysis described in chapter 5.0 will allow the design of diagnostic PCR primers or probes for the rapid and specific identification of the aetiological agent of granuloma inguinale. The ability to culture, characterise and identify the organism at a molecular level will help to elucidate the pathogenesis and virulence factors of *C. granulomatis* which are presently unknown.

CHAPTER 7.0
CONCLUSIONS

7.1 This study describes the first ever method for the cultivation of *Calymmatobacterium granulomatis* using a monocyte co-culture system.

7.2 The ultrastructural anlysis of *C. granulomatis* demonstrated

7.2.1 a similar capsule morphology from monocyte co-cultures and tissue biopsy specimens which was electron dense and not electron lucent as described in previous reports.

7.2.2 a characteristic Gram negative cell wall

7.2.3 the absence of phage particles, fimbriae and flagella in the organism.

7.3 With regard to the molecular analysis

7.3.1 bacterial DNA was successfully amplified, cloned and sequenced from the monocyte co-culture, frozen and formalin fixed paraffin wax embedded tissue biopsy specimens

7.3.2 hybridisation with specific probes confirmed that the 16S rDNA sequences derived from all three sources (monocyte co-culture, frozen and paraffin wax embedded tissue biopsy) were Eubacterial and Gram negative in origin.

7.3.3 on the basis of the 16Sr DNA analysis *Calymmatobacterium granulomatis* was closely related to, but distinct from the *Klebsiella* group

7.3.4 *Calymmatobacterium* belongs to the gamma subclass of *Proteobacteria* group

7.3.5 confirms the validity of the present genus *Calymmatobacterium* within the taxon *Enterobacteriaceae*

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APPENDICES

APPENDIX A: REAGENTS AND SOLUTIONS USED FOR CELL CULTURE

Histopaque 1077

100ml available from Sigma Diagnostics, St Louis MA, USA.

Hanks balanced salt solution

500ml volume with glutamine, catalogue number 12-702F available from Biowhittaker, Walkersville, Maryland, USA.

RPMI 1640

500ml volume with glutamine, catalogue number 12-702F available from Biowhittaker, Walkersville, Maryland, USA.

Foetal Calf Serum

500ml volume, endotoxin less than 2EU/ ml, catalogue number 14501 BI available from Delta Bioproducts, South Africa. Complement was inactivated at 56°C for 30 minutes. The sera was aliquoted into 50ml amounts and kept frozen until use.

Antibiotic stock solutions

Amikacin (final concentration 10μg/ml) - 2000mg amikacin (was dissolved in 20ml sterile distilled water.

Vancomycin (final concentration 5μg/ml) - 1000mg vancomycin was dissolved in 20mls of sterile water.

Metronidazole (final concentration 10μg/ml) - 2000 mg metronidazole was dissolved in 20 mls of sterile distilled water.

The antibiotic solutions were distributed in 1ml amounts separately and stored at -70°C. One aliquot of each was added to 500mls of medium where required to give the appropriate final concentration.

RapiDiff Stains

Avaliable from Clinical Science Diagnostics, South Africa.

Trypan Blue (vital stain, CI 23850)

400mg was added to 90 mls water containing 810mg NaCl, 60 mg K_2 HPO_4, and 50 mg methyl *p* - hydroxybenzoate. The mixture was boiled, cooled and the pH adjusted to 7,2 with 1 N NaOH. The final volume was adjusted to 100mls.

APPENDIX B: SOLUTIONS AND REAGENTS FOR TRANSMISSION ELECTRON MICROSCOPY

1% Glutaraldehyde

Glutaraldehyde (1ml) was added to 100 ml of Eagles minimal essential media (Biowhittaker, Walkersville, Maryland, USA).

1% Osmium tetroxide

Osmium tetroxide (0,1g) was dissolved in 10 ml cacodylate buffer.

Araldite epoxy resin

Alcohol - 70%; 90% and 100%

APPENDIX C: SOLUTIONS AND REAGENTS USED FOR DNA EXTRACTION

0.5 M EDTA (pH 8)

186.1 g of disodium EDTA.$2H_2O$ was dissolved in 800 ml of water; stirred vigorously; the pH adjusted to 8 with approximately 20 g of NaOH pellets, and the volume made to 1,000 ml with water. The solution was autoclaved, and stored at room temperature.

1 M Tris-HCI (pH 8)

121.1 g of Tris base was dissolved in 800 ml of water; 42 ml of concentrated HCI was added. The volume made up to 1000 ml. The solution was autoclaved and stored at room temperature.

TE buffer

10 mM Tris-HCI (pH 8.0), 1 mM EDTA (pH 8). For 100 ml of TE : 1 ml of 1 M Tris - HCI (pH 8) and 0.2 ml of 0.5 M EDTA (pH 8) and made up to 100 ml with water, filter sterilized and stored at room temperature.

5 x TBE buffer (Tris-borate-EDTA)

5 x 27.5 g of boric acid, 54.0 g of Tris base, 20,0 ml of 0,5 M EDTA (pH 8); was added to 1000 ml of water; stored at room temperature and when required diluted to working concentration with water.

3 M NaCI

175.32 g of NaCI was dissolved in 800 ml of water using low heat and stirring to aid dissolution; the volume was made up to 1000 ml, autoclaved and stored at room temperature.

10% sodium dodecyl sulfate (SDS)

10g of SDS was dissolved in 80 ml of water; the volume made up to 100 ml. The solution was filter sterilised and stored at room temperature.

Proteinase K (Boehringer Mannheim, Germany)

20 mg/ml in water; dispensed into 500µl aliquots into 0.5 ml Eppendorf tubes; stored at -20^{0}C.

Phenol : chloroform : isoamyl alcohol (25 : 24 : 1)

Saturated with 10mM Tris pH 8,0 1mM EDTA available from Sigma (P 2069)

Chloroform-isoamyl-alcohol

24 volumes of chloroform, 1 volume isoamyl alcohol; stored in the dark bottle at +4^{0}C.

95% ethanol : stored at +4^{0}C.

Ribonuclease A (Boehringer Mannheim, Germany)

Stock solution

10 mg/ml in water; 1 mg of RNAse A was dissolved in 10ml of water and boiled for 10 min. 100µl was dispensed into 0.5 ml Eppendorf tubes; stored at -20^0 C.

Ethidium bromide (E 8751 Sigma)

Stock solution

10 mg/ml; 1 g of ethidium bromide was dissolved in 100 ml of water; the solution was stirred with a magnetic stirrer for several hours; filtered through a Whatman no. 1 membrane to remove residue; transferred to a dark bottle and stored at +4^{0}C.

Working solution

0,5 µg/ml

10 x gel-loading buffer

0,25% bromphenol blue, 0,25% xylene cyanol and 30% glycerol in water; stored at room temperature.

Agarose

Depending on the strength of the required gel, NuSieve agarose (FMC BioProducts, USA)

was weighed and dissolved in 1x TBE buffer. This was heated in a microwave until the agarose dissolved. The agarose solution was cooled to 50°C and ethidium bromide added (final concentration 0,5µg / ml (stock 10 mg / ml). The agarose was gently mixed and poured into casting trays.

APPENDIX D: SOLUTIONS AND REAGENTS FOR PCR

Magnesium chloride (5mM)

1µl of 1M $MgCl_2$ (Sigma) was diluted with 199µl of sterile distilled water, aliquoted and stored at -20^{0}C. 10µl was used in 100µl amplification reaction to give a final concentration of 5mM.

PCR buffer

A 10x concentration was available from Boehringer Mannheim which consisted of Tris HCl 100mM; $MgCl_2$ 15mM; KCl 500mM; gelatine 0,1mg/ml.

Amplification primers

P1F was diluted 1 : 24; P4R was diluted 1 : 23. 1µl of each primer was used in 100 µl amplification reaction to give a final concentration of 20 pmol/µl.

***Taq* DNA polymerase (5U/µl) (Boehringer Mannheim, Germany)**

0,5µl was used in a 100µl amplification reaction.

dNTP mix - 100mM

dATP (Boehringer Mannheim, Germany)	1µl
dCTP (Boehringer Mannheim, Germany)	1µl
dGTP (Boehringer Mannheim, Germany)	1µl
dTTP (Boehringer Mannheim, Germany)	1µl
dH_20	16µl

This mixture was aliquoted and stired at -20^{0}C. 2µl of each dNTP mix in a 100µl volume was used to give the final concentration of 10mM of each dNTP.

APPENDIX E: REAGENTS AND MEDIA USED FOR CLONING AND TRANSFORMATION OF PCR PRODUCTS

Lauria Bertonni (LB) agar plates with antibiotics

In a flask tryptone 10g, yeast extract 5g, NaCl 10g and agar 15g were dissolved in 1000ml of distilled water and sterilised by autoclaving. The medium was cooled to 50°C and freshly prepared ampicillin (50 μg/ml) and tetracycline (15 μg/ml) were added. The surface moisture was allowed to dry from plates before use. This medium was used to select recombinant clones after the spreading of X-gal and IPTG.

Lauria Bertonni (LB) medium

In a flask tryptone 10g, yeast extract 5g and NaCl 10g were dissolved in 1000ml of distilled water and sterilised by autoclaving. The medium was stored at 4°C. When required freshly prepared ampicillin (50 μg/ml) was added and this medium was used to select and grow recombinant clones for plasmid extraction.

SOC medium

In a flask bacto-tryptone 20g, bacto- yeast extract 5g, NaCl 0,5g was dissolved in 950 mls of distilled water. 10 ml of a 250mM solution of KCl was added and pH adjusted to 7,0 with 5N NaOH. The volume was adjusted to 975 mls and sterilized by autoclaving. Just before use 5 mls of sterile 2M MgCl2 and 20 mls of a 1M solution of glucose was added.

Isopropyl β-D-thiogalactopyranoside (IPTG) (Boehringer Mannheim, Germany)

100mM solution

2g of IPTG was dissolved in 10ml sterile distilled water. The solution was filter sterilised, aliquoted into 1ml amounts and stored in the dark at 4°C.

5-Bromo-4-chloro-3-indolyl-β-D-galactoside(X-gal)(Boehringer Mannheim, Germany)

A 50mg/ml X-gal solution was prepared in dimethylformamide and stored in the dark at 4°C.

APPENDIX F: SOLUTIONS AND REAGENTS USED FOR HYBRIDISATION AND DETECTION

20 x SSC

3M NaCl

0,3M Sodium citrate , pH 7.0

175.3 g of NaCI and 88.2 g of sodium citrate was dissolved in 800 ml of water; the pH adjusted to 7 with a few drops of a 10 N NaOH and the volume made up to 1,000 ml; autoclaved and stored at room temperature.

Buffer 1

150 mmol/l NaCI

100 mmol/l maleic acid, pH 7.5

Adjusted with solid or concentrated NaOH, autoclaved.

Blocking Reagent stock solution (10% w/v)

10g of blocking reagent was dissolved in 100 ml maleic acid buffer with gentle heating. The solution was autoclaved and stored at 4^0C. The solution was checked for contamination before each use.

Buffer 2

Blocking reagent stock solution diluted 1 : 10 in buffer 1 (final concentration = 1% Blocking Reagent).

Buffer 3

100 mmol/l Tris-HCI, pH 9.5

100 mmol/l NaCI

50 mmol/l $MgCI_2$.

TE buffer

10 mmol/l Tris-HCI, pH 8 (+25^{0}C)

1 mmol/l EDTA

Denaturation solution 1

0.5 N NaOH

1.5 mol/l NaCI.

Neutralization solution 1

0.5 mol/l Tris - HCI, pH 7.5

3 mol/l NaCI

Washing solution 2 x

2 x SSC

0.1% SDS

Washing solution 0.1 x

0.1 x SSC

0.1% SDS

APPENDIX G: SOLUTIONS AND BUFFERS FOR SEQUENCING WITH T7 DNA POLYMERASE

NaOH

1M stock solution aliquoted in 1ml batches to prevent transient neutralisation of the NaOH by atmospheric CO2.

HCl

1M stock solution aliquoted in 1ml batches.

Annealing buffer

1M Tris HCl, pH8, 100mM $MgCl_2$

Labelling Mix

10 μM fluorescein-15-*dATP, 1 μM each of dCTP, dGTP, dTTP

Extension buffer

40mM $MnCl_2$, 304mM NaCitrate, 324mM DTT

Termination mixes

1mM dATP, 1mM dCTP, 1mM c^7dGTP, 1mM dTTP, 5 μM ddNTP, 50mM NaCl, 40mM Tris-HCl (pH7.4)

Enzyme

T7 DNA polymerase (Pharmacia LKB Biotechnology, Inc)

APPENDIX H: ELECTROPHEROGRAMS OF SEQUENCING RESULTS

APPENDIX H (i): ELECTROPHEROGRAM FOR CLONE KH 22

Model 377
Version 2.1.1

20•22.M13F

22.M13F
Lane 20

Signal G:92 A:223 T:95 C:90
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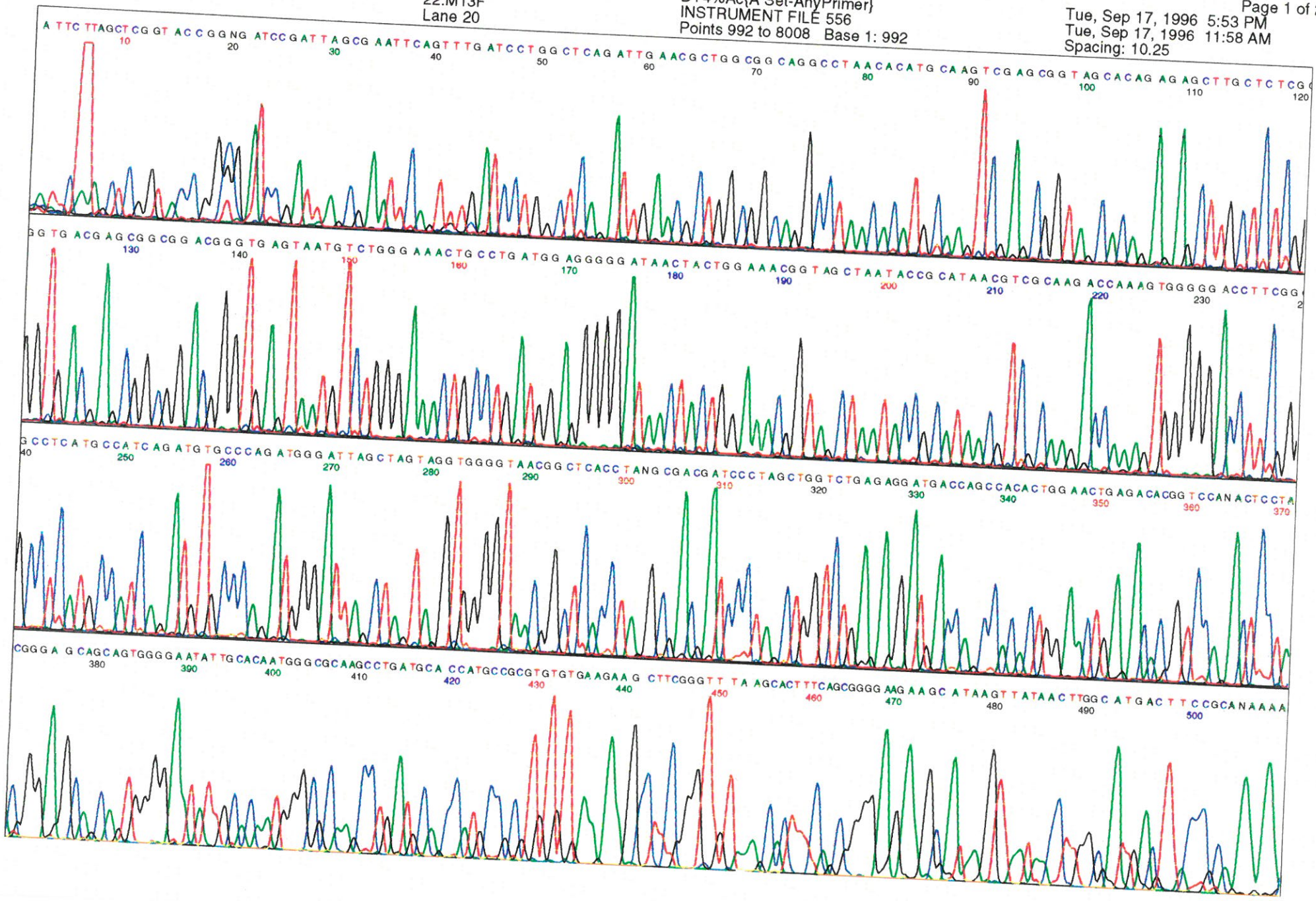

Model 377
Version 2.1.1

20•22.M13F

22.M13F
Lane 20

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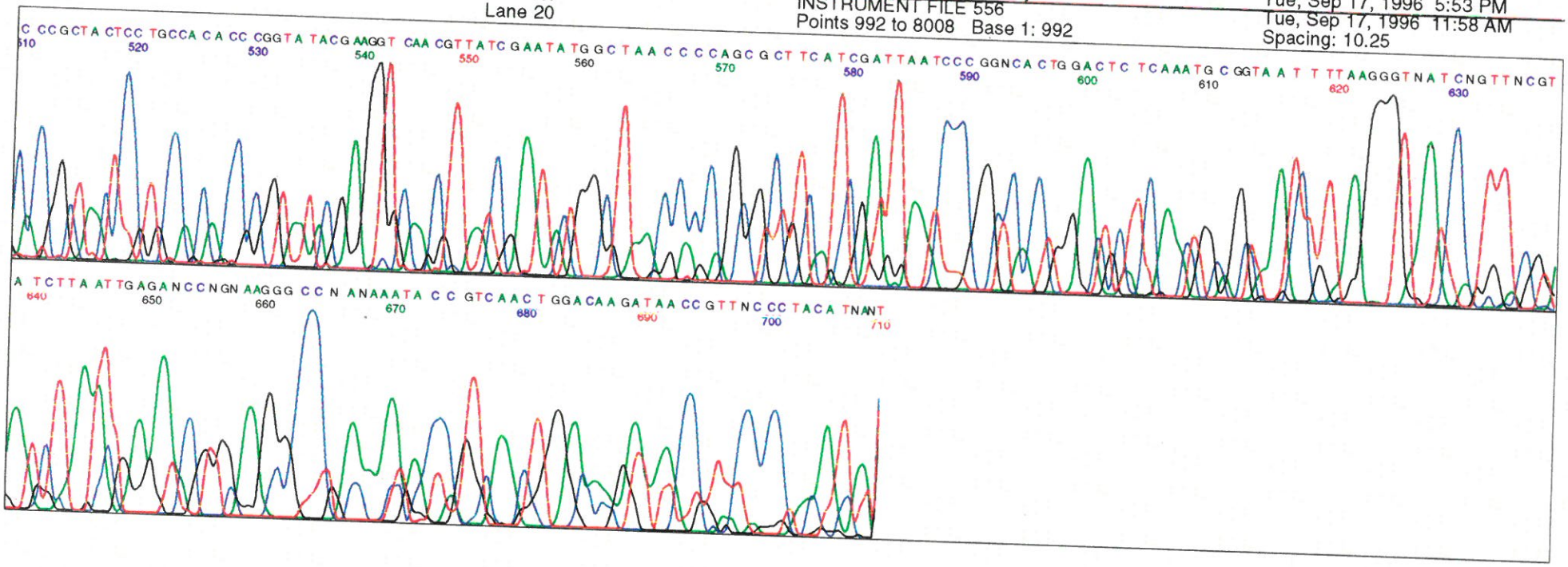

Applied Biosystems

Model 377
Version 2.1.1

18•22.U19

22.U19
Lane 18

Signal G:151 A:264 T:79 C:75
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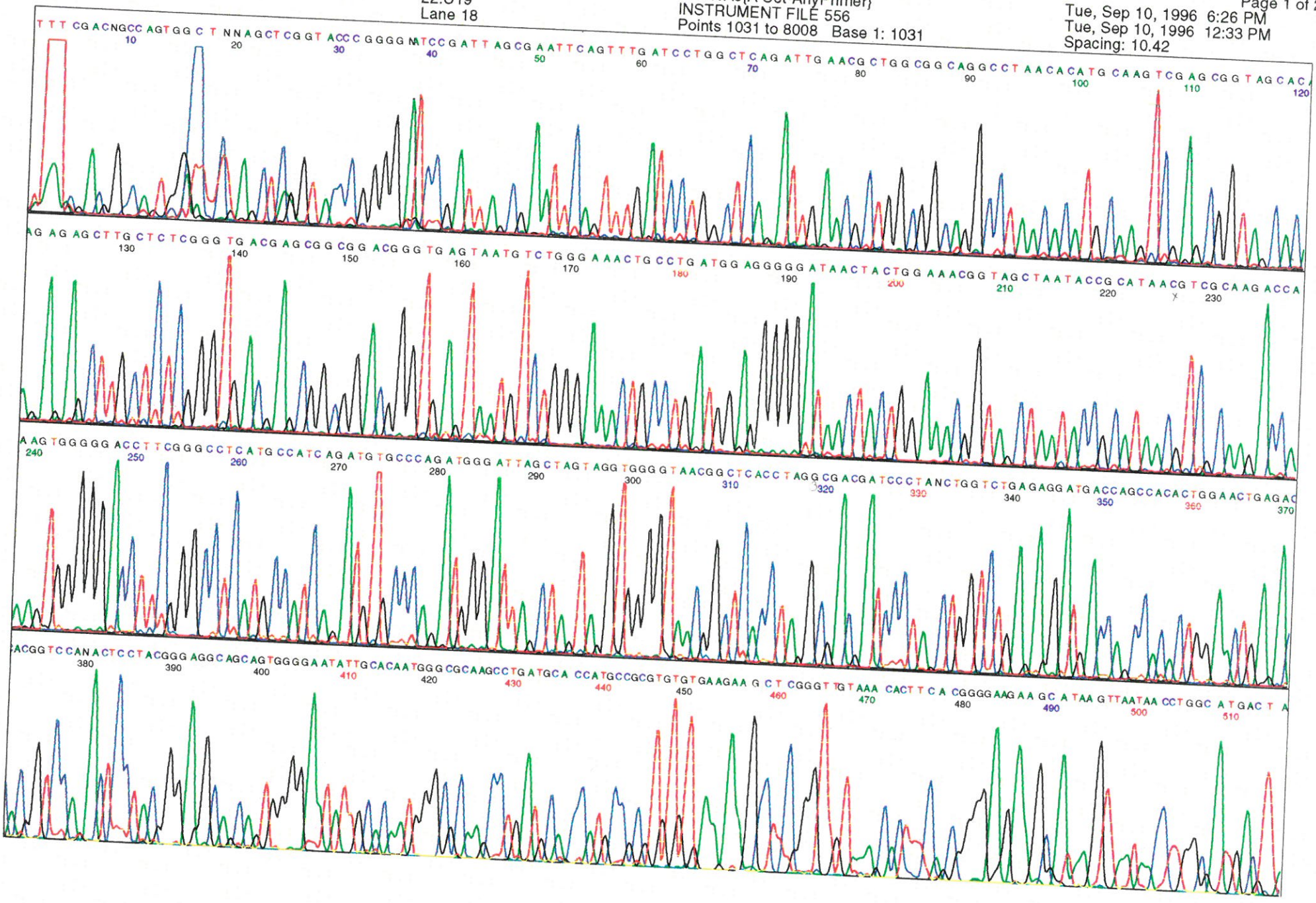

Model 377
Version 2.1.1

18•22.U19

22.U19
Lane 18

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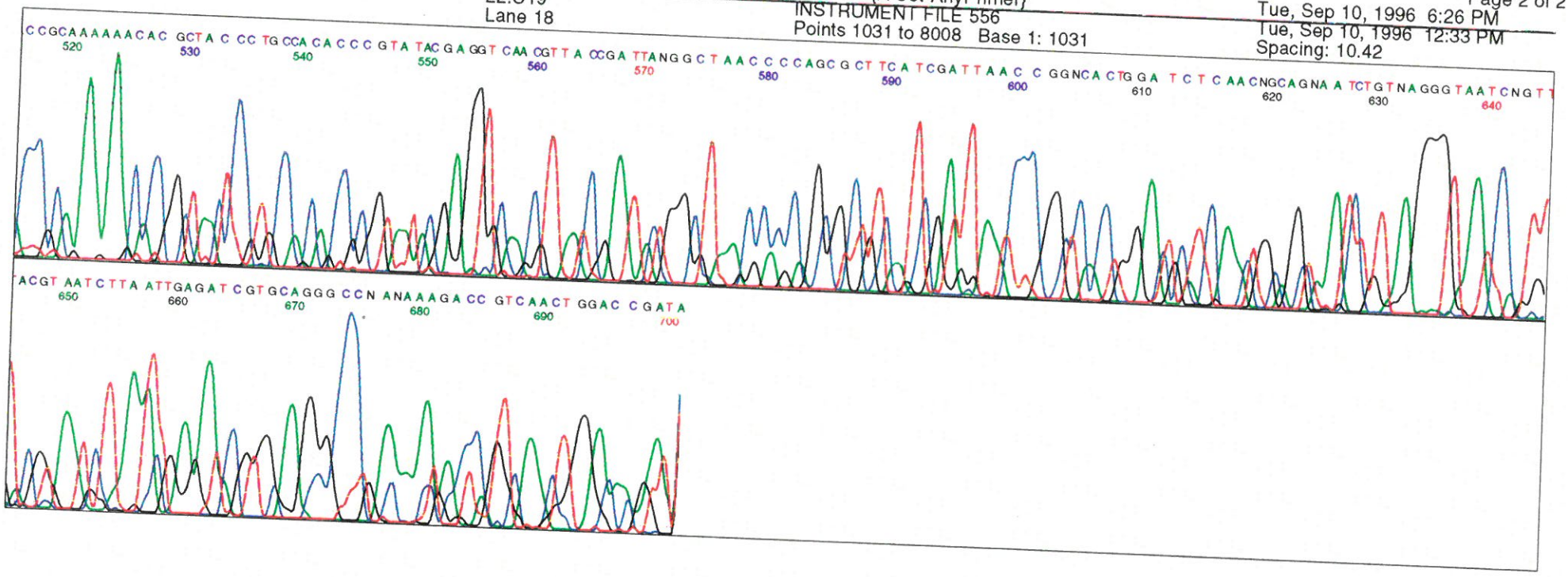

Applied Biosystems

Model 377
Version 2.1.1

10•22.61.1

22.61
Lane 10

Signal G:321 A:543 T:283 C:361
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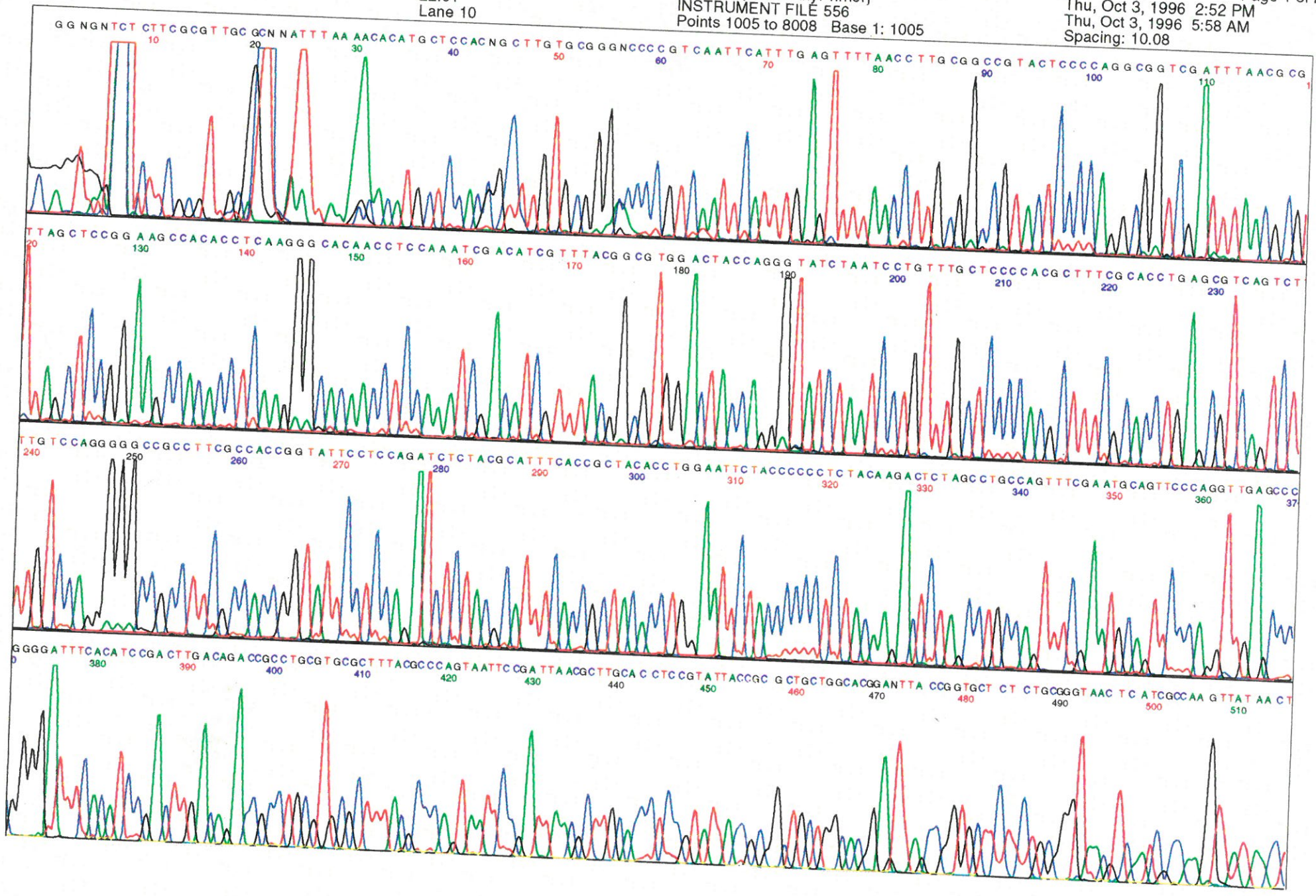

Model 377
Version 2.1.1

10•22.61.1

22.61
Lane 10

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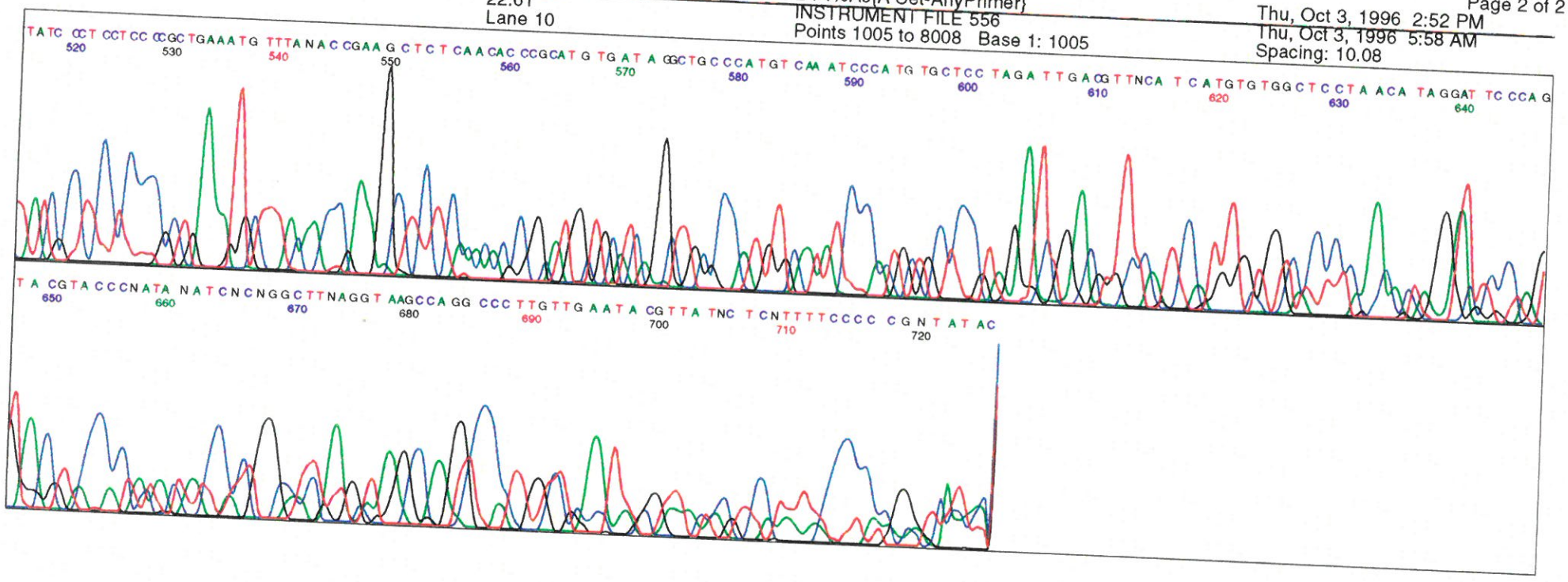

Applied Biosystems

Model 377
Version 2.1.1

13•22.62.1

22.62
Lane 13

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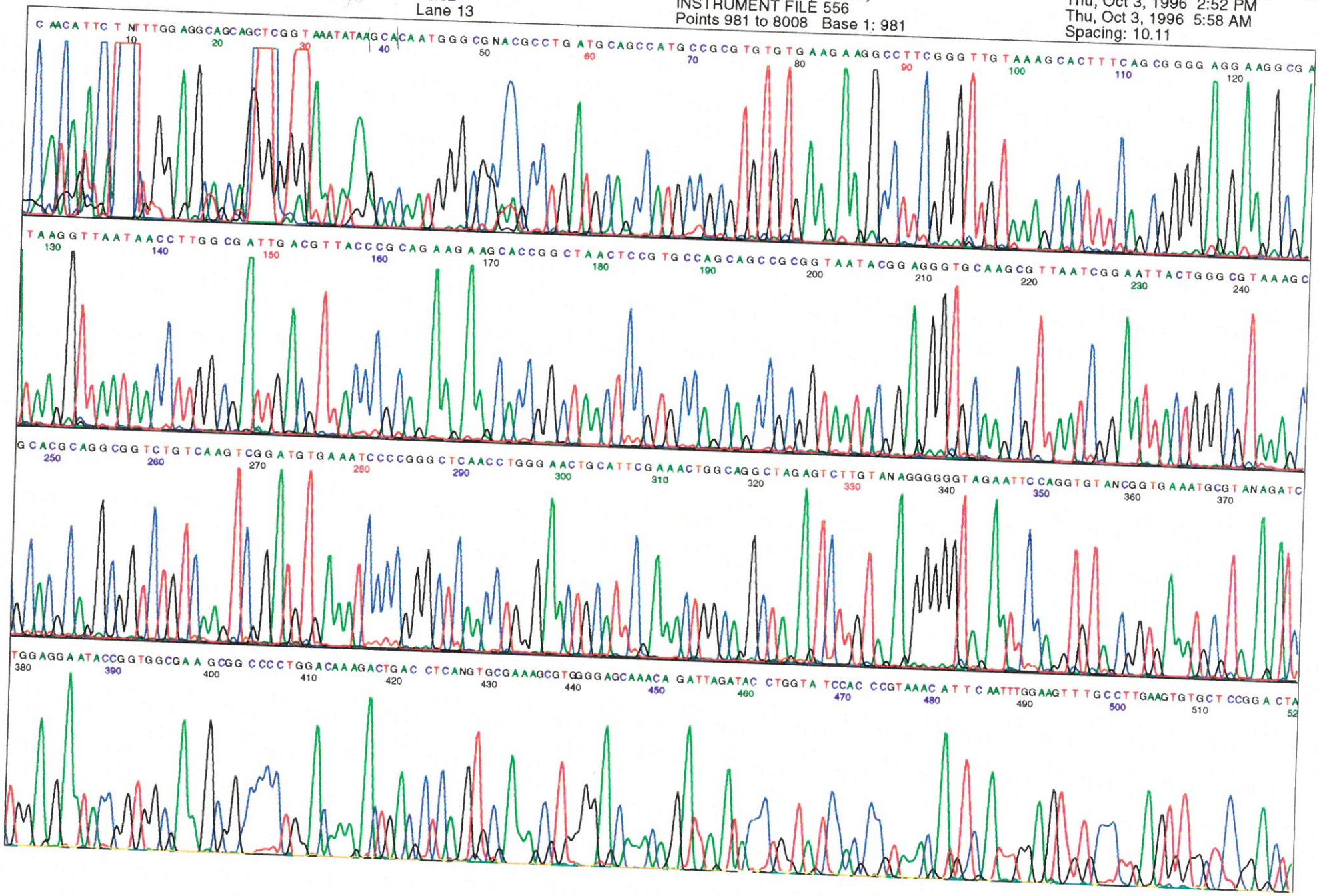

Model 377
Version 2.1.1

13•22.62.1

22.62
Lane 13

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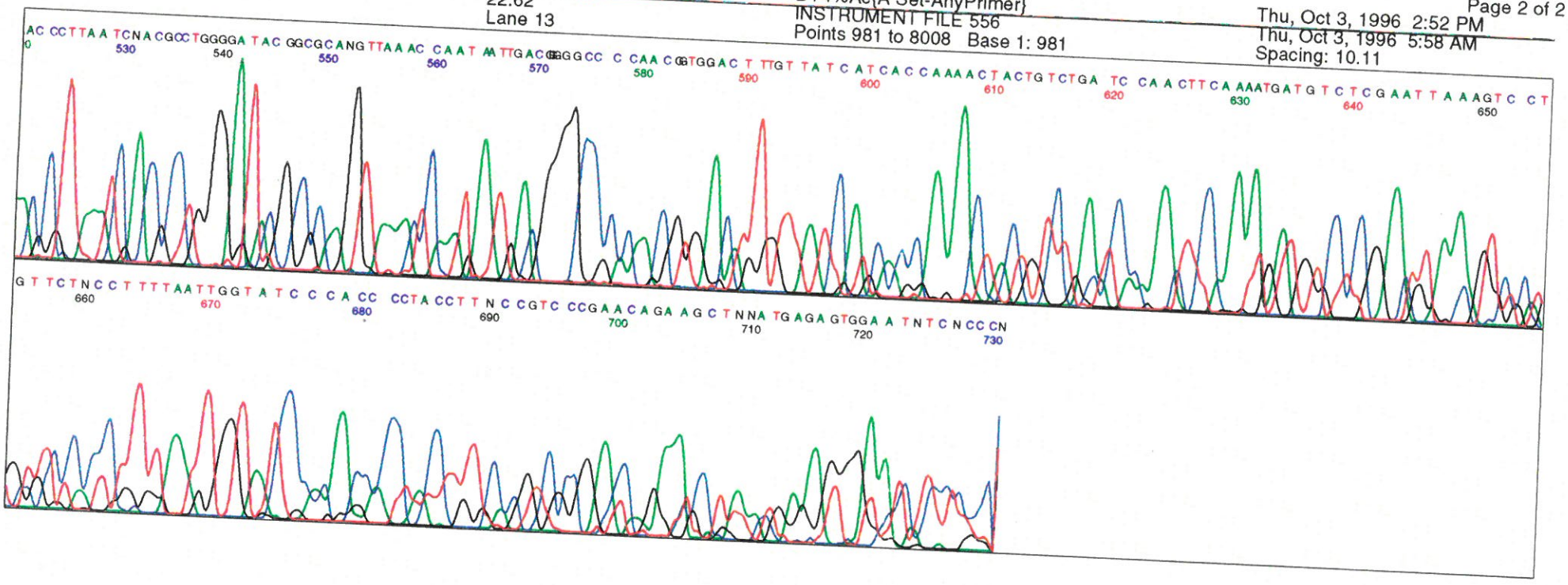

Applied Biosystems

Model 377
Version 2.1.1

16•22.MOS1.1

22.MOS1
Lane 16

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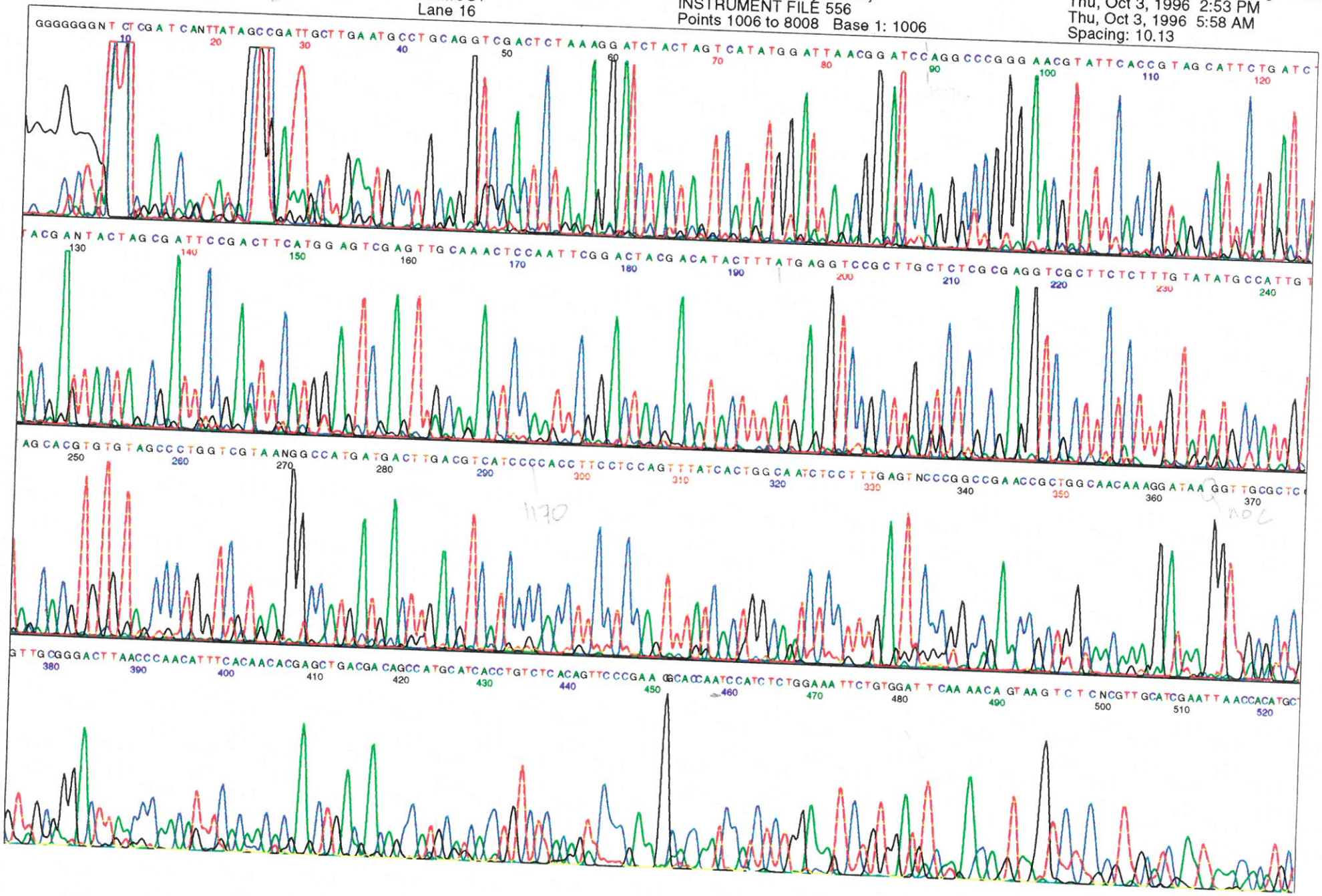

Model 377
Version 2.1.1

16•22.MOS1.1

22.MOS1
Lane 16

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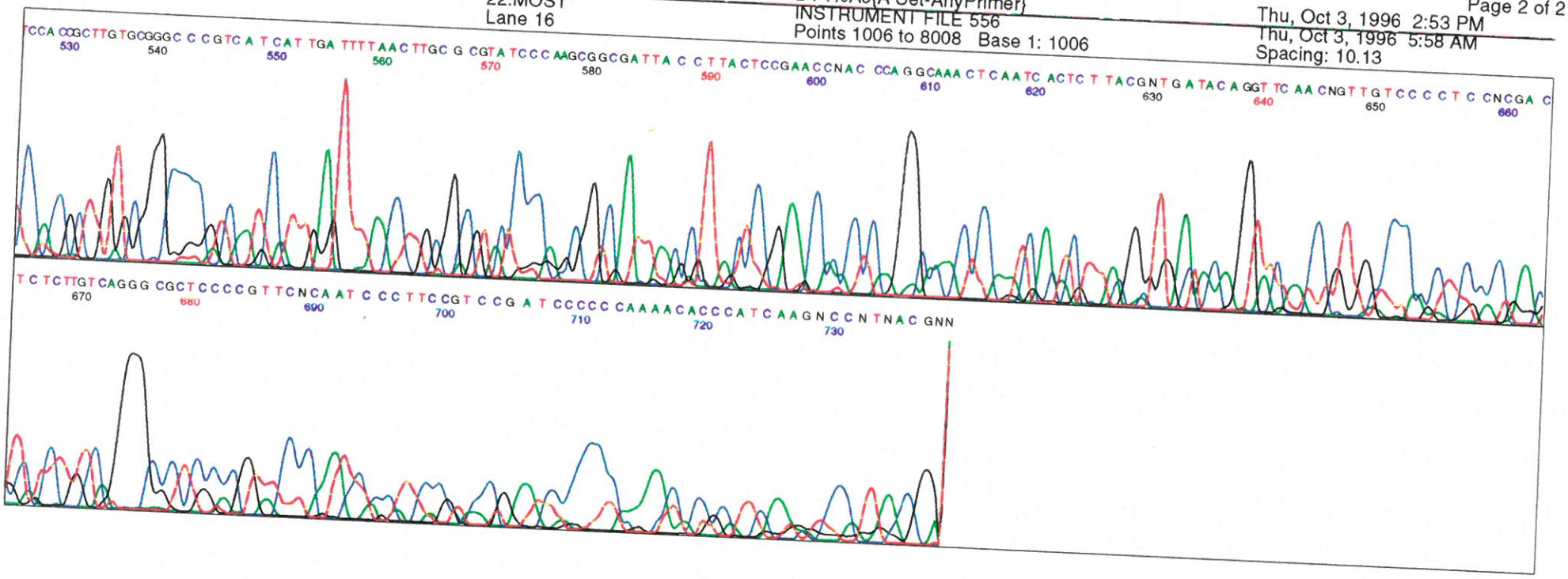

APPENDIX H (ii): ELECTROPHEROGRAM FOR CLONE KH 6

Applied Biosystems

Model 377
Version 2.1.1

19•6.M13F

6.M13F
Lane 19

Signal G:70 A:110 T:39 C:37
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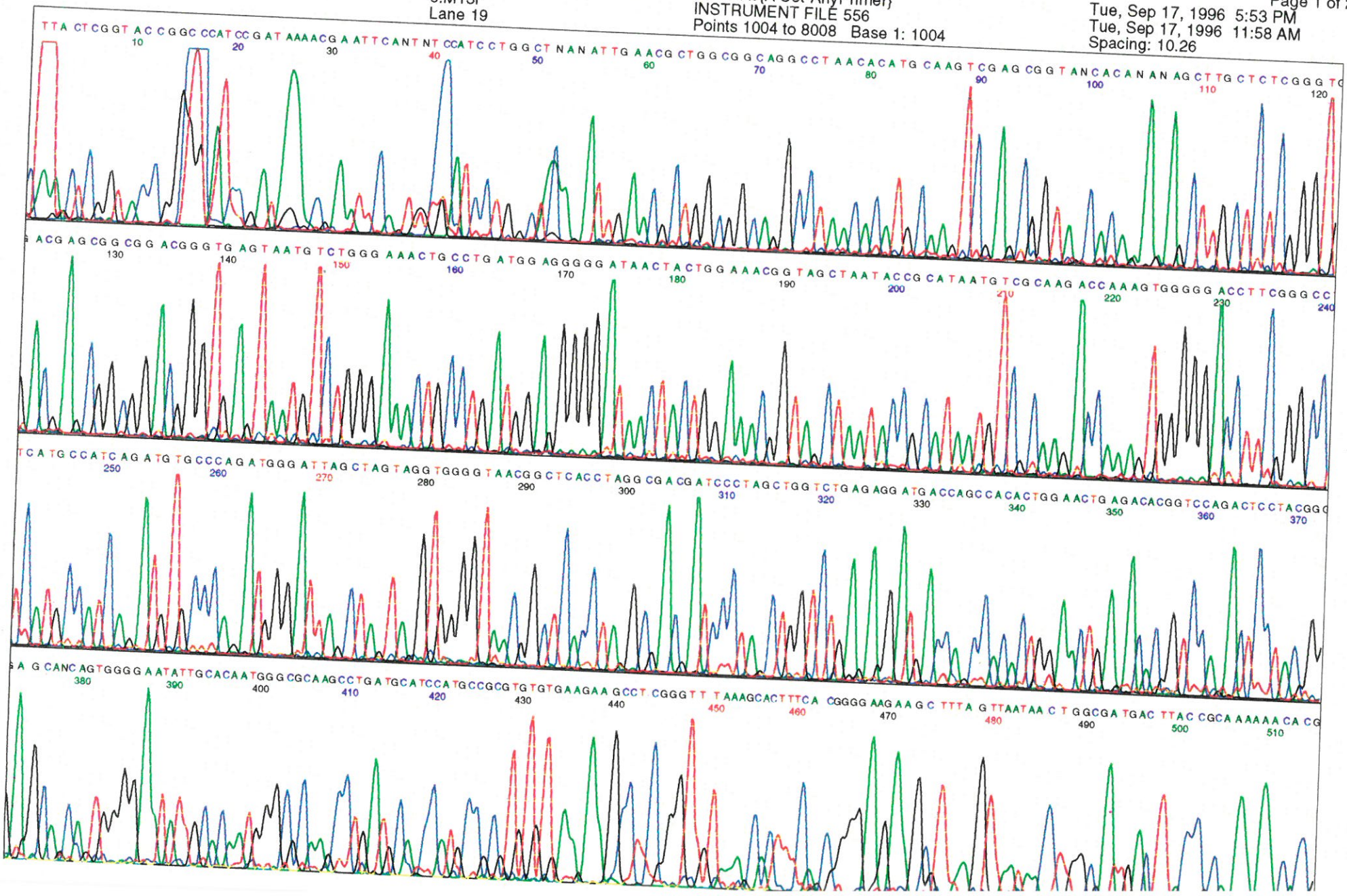

Applied Biosystems

Model 377
Version 2.1.1

19•6.M13F

6.M13F
Lane 19

Signal G:70 A:110 T:39 C:37
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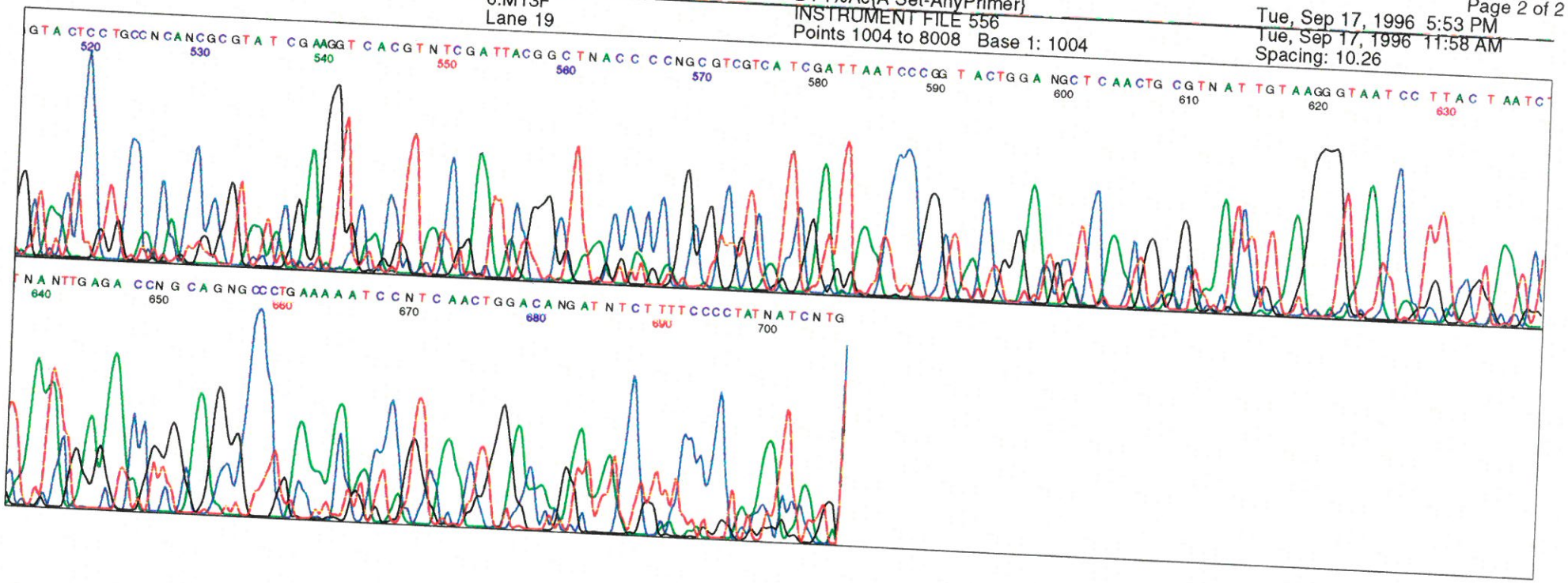

Applied Biosystems

Model 377
Version 2.1.1

15•6.U19

6.U19
Lane 15

Signal G:106 A:145 T:48 C:38
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Points 1024 to 8008 Base 1: 1024

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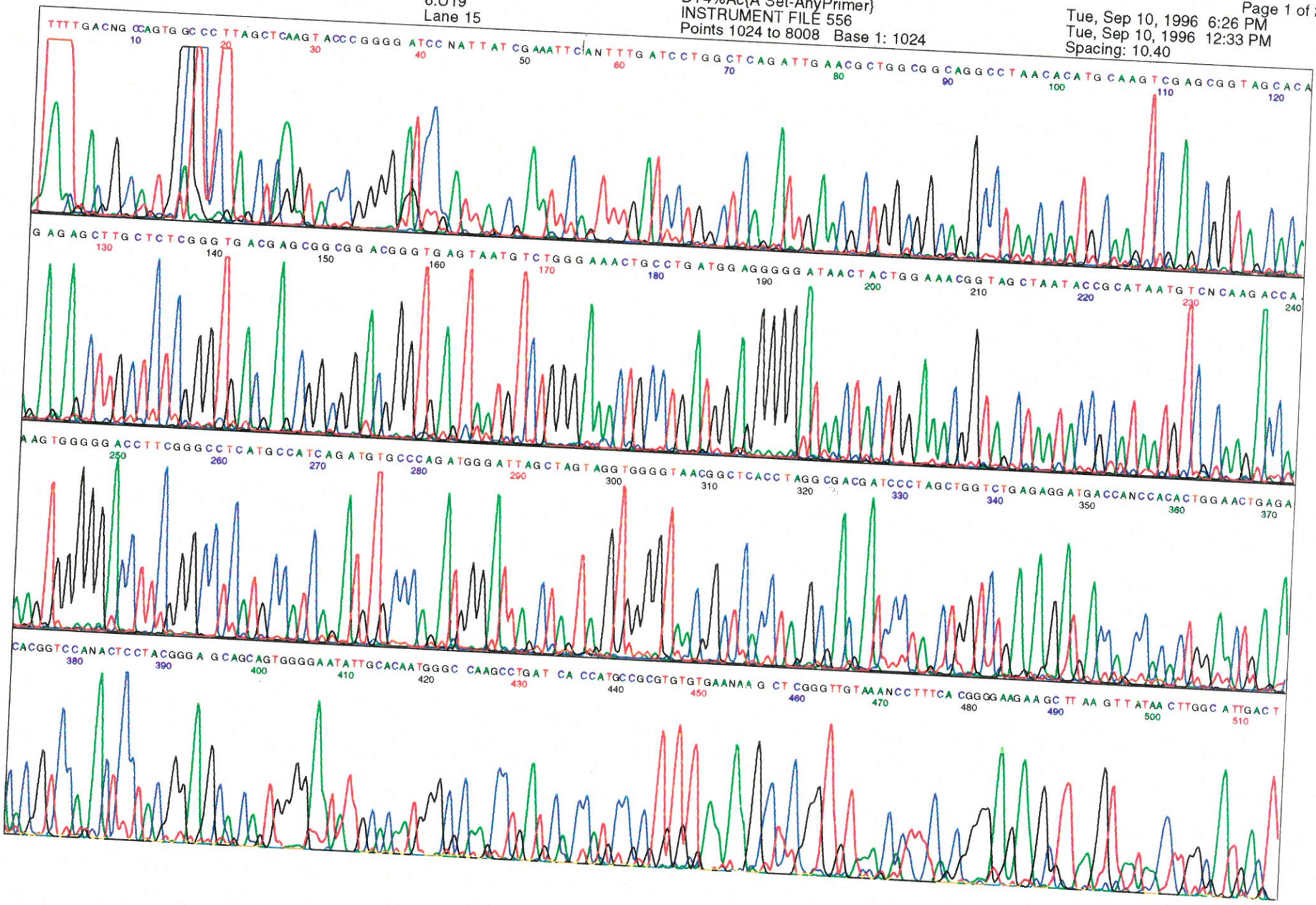

Applied Biosystems

Model 377
Version 2.1.1

15•6.U19

6.U19
Lane 15

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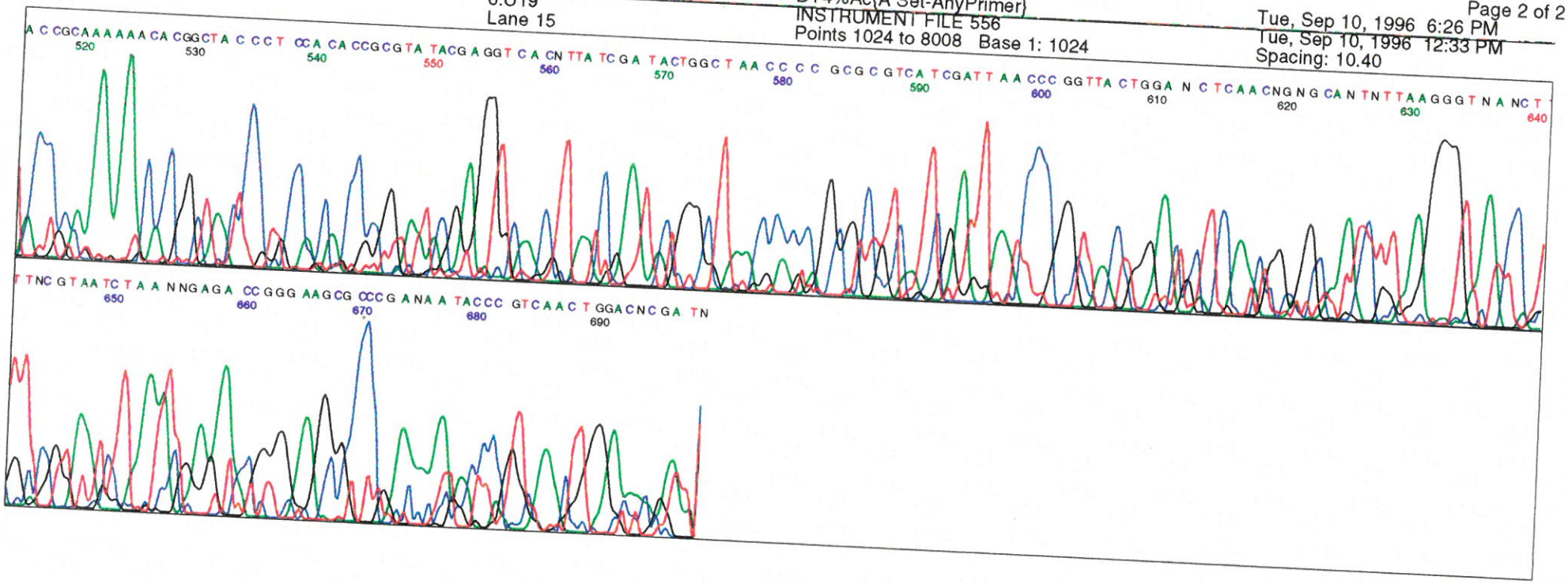
Tue, Sep 10, 1996 6:26 PM
Tue, Sep 10, 1996 12:33 PM
Spacing: 10.40



Applied Biosystems

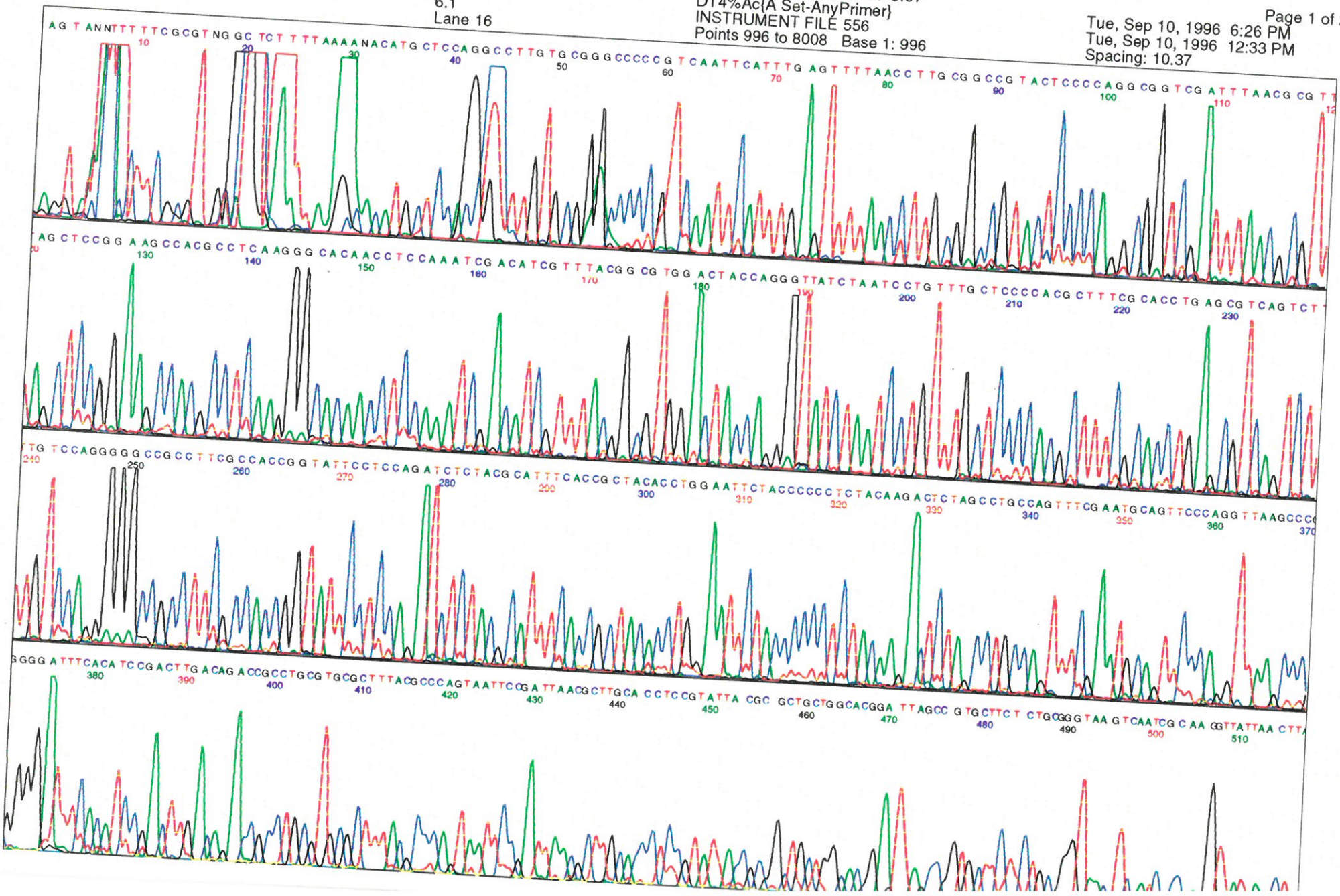
Model 377
Version 2.1.1

16•6.1

6.1
Lane 16

Signal G:110 A:139 T:109 C:97
DT4%Ac{A Set-AnyPrimer}
INSTRUMENT FILE 556
Points 996 to 8008 Base 1: 996

Tue, Sep 10, 1996 6:26 PM
Tue, Sep 10, 1996 12:33 PM
Spacing: 10.37



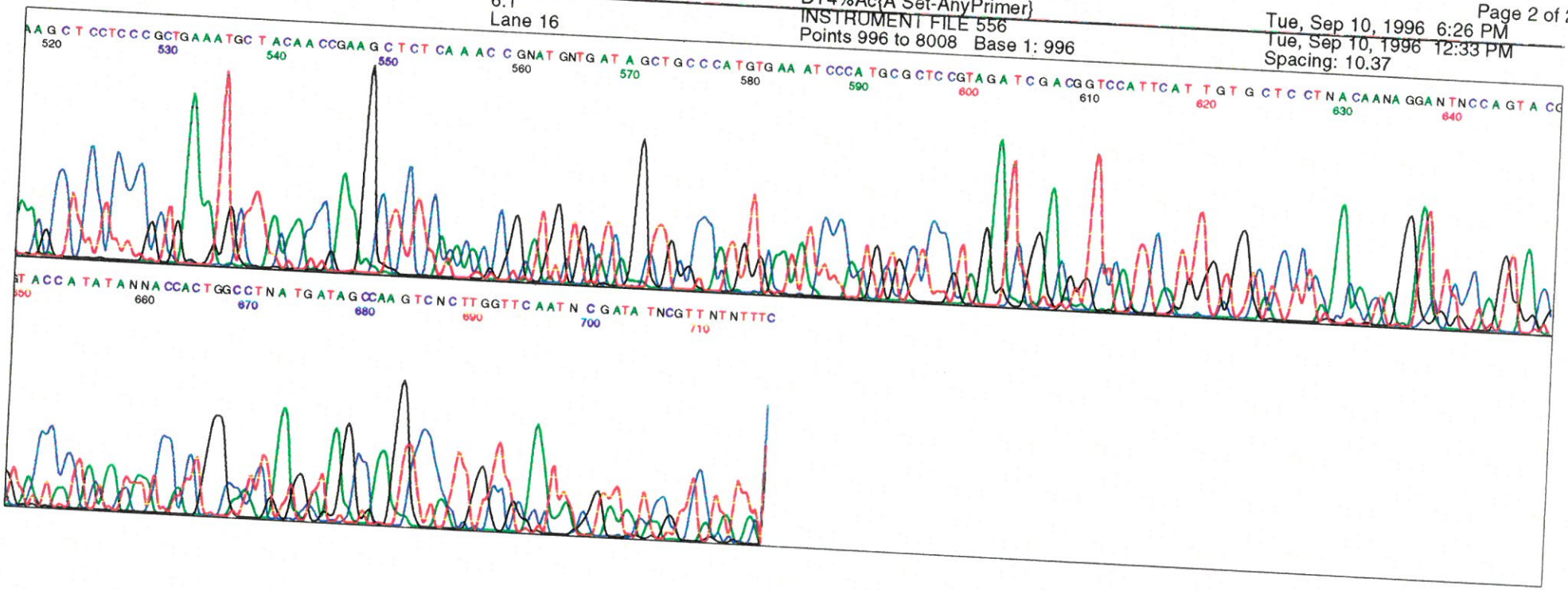
Model 377
Version 2.1.1

16•6.1

6.1
Lane 16

Signal G:110 A:139 T:109 C:97
DT4%Ac{A Set-AnyPrimer}
INSTRUMENT FILE 556
Points 996 to 8008 Base 1: 996

Tue, Sep 10, 1996 6:26 PM
Tue, Sep 10, 1996 12:33 PM
Spacing: 10.37



Applied Biosystems

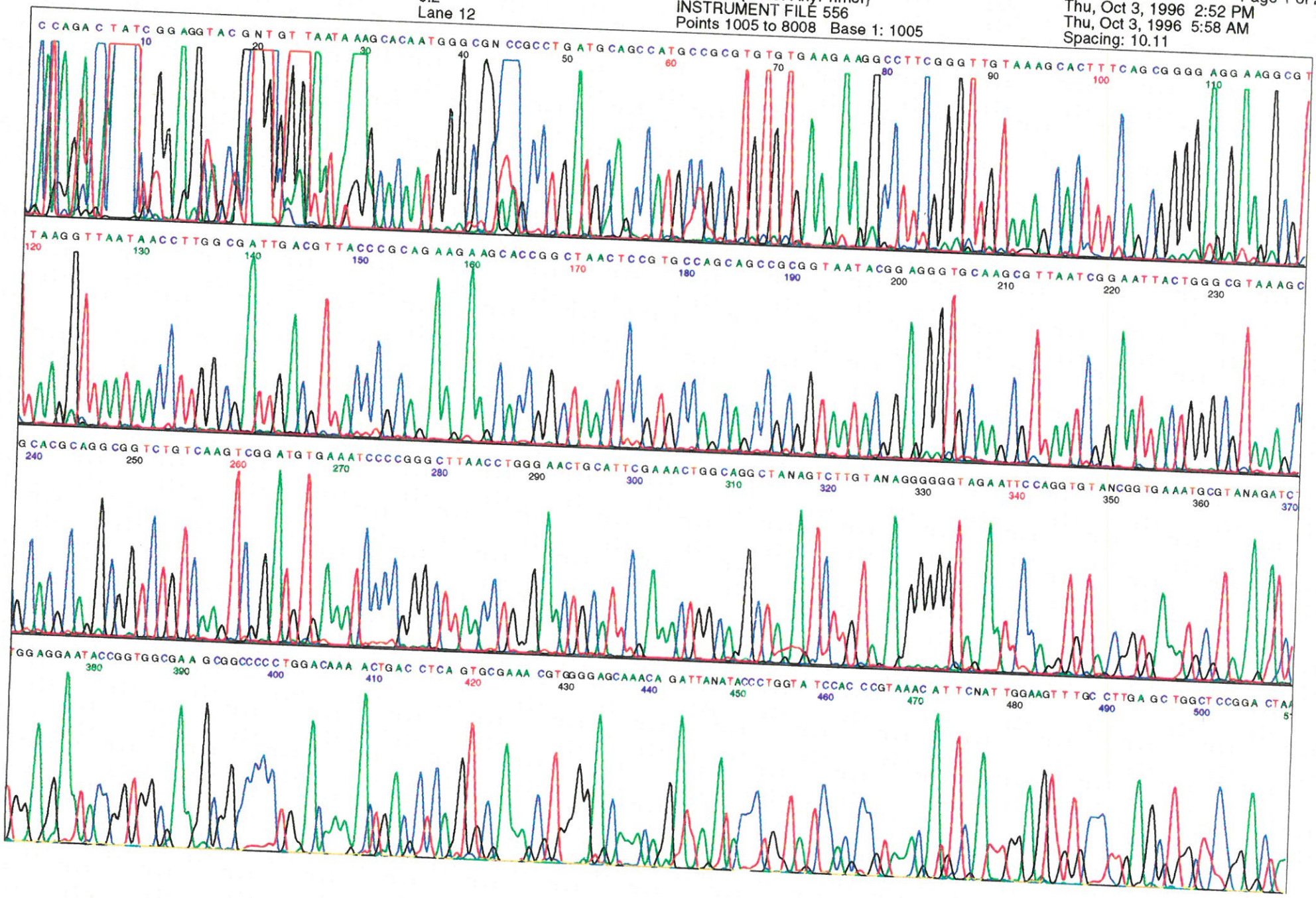
Model 377
Version 2.1.1

12•6.2.1

6.2
Lane 12

Signal G:423 A:629 T:251 C:238
DT4%Ac{A Set-AnyPrimer}
INSTRUMENT FILE 556
Points 1005 to 8008 Base 1: 1005

Thu, Oct 3, 1996 2:52 PM
Thu, Oct 3, 1996 5:58 AM
Spacing: 10.11



Applied Biosystems

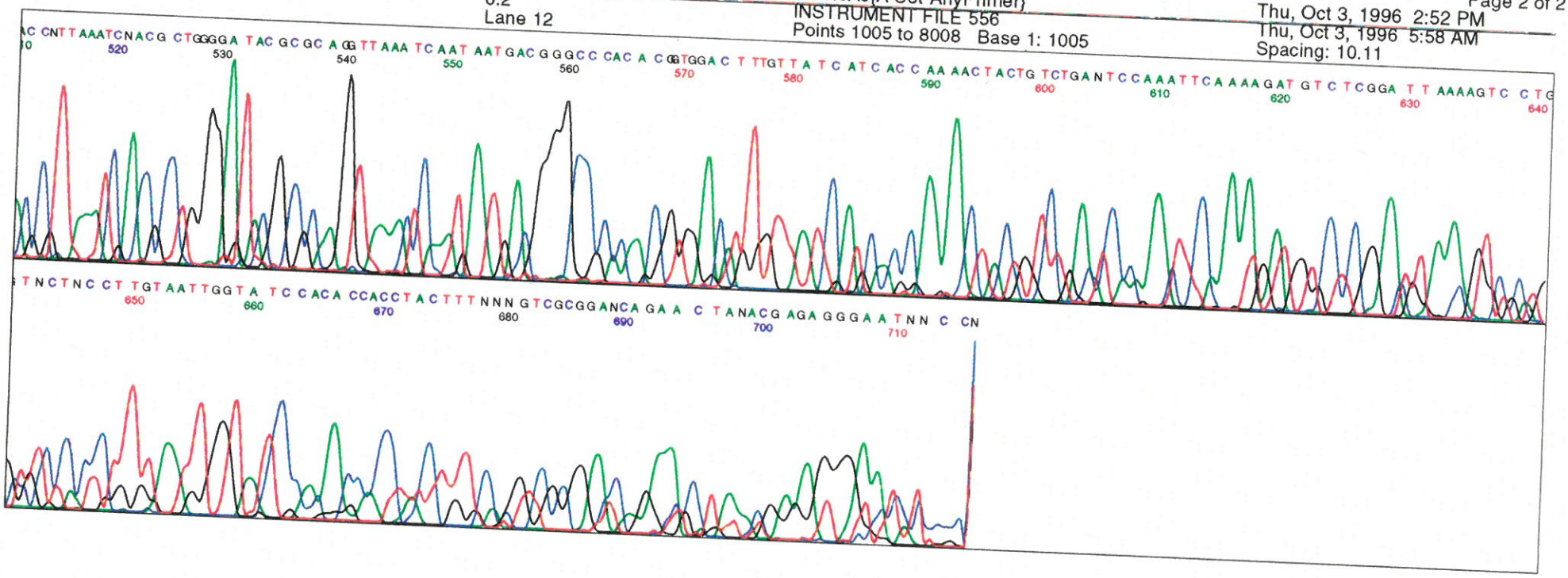
Model 377
Version 2.1.1

12•6.2.1

6.2
Lane 12

Signal G:423 A:629 T:251 C:238
DT4%Ac{A Set-AnyPrimer}
INSTRUMENT FILE 556
Points 1005 to 8008 Base 1: 1005

Thu, Oct 3, 1996 2:52 PM
Thu, Oct 3, 1996 5:58 AM
Spacing: 10.11



Reverse

Applied Biosystems

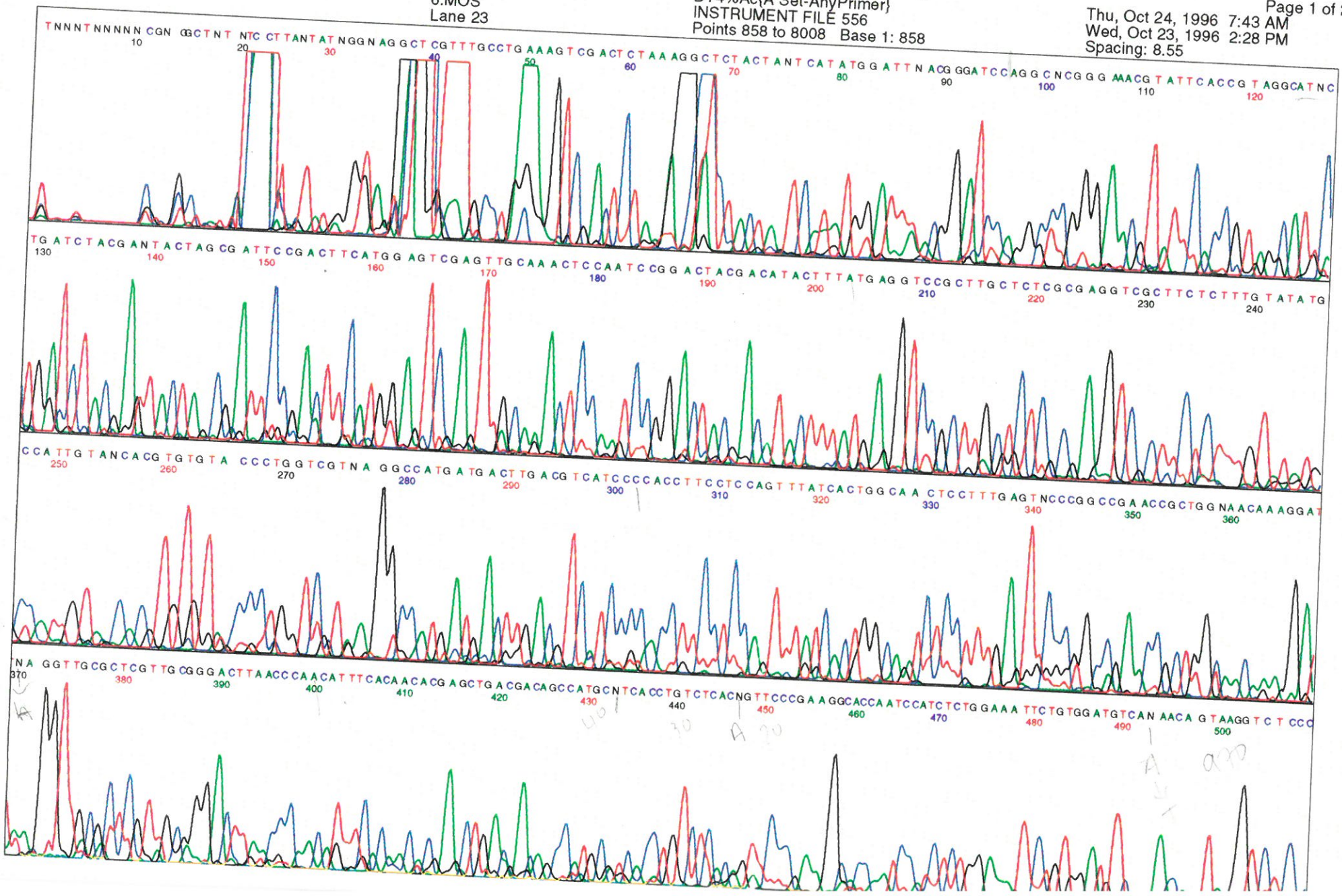
Model 377
Version 2.1.1

23•6.MOS.1

6.MOS
Lane 23

Signal G:285 A:430 T:176 C:189
DT4%Ac{A Set-AnyPrimer}
INSTRUMENT FILE 556
Points 858 to 8008 Base 1: 858

Thu, Oct 24, 1996 7:43 AM
Wed, Oct 23, 1996 2:28 PM
Spacing: 8.55



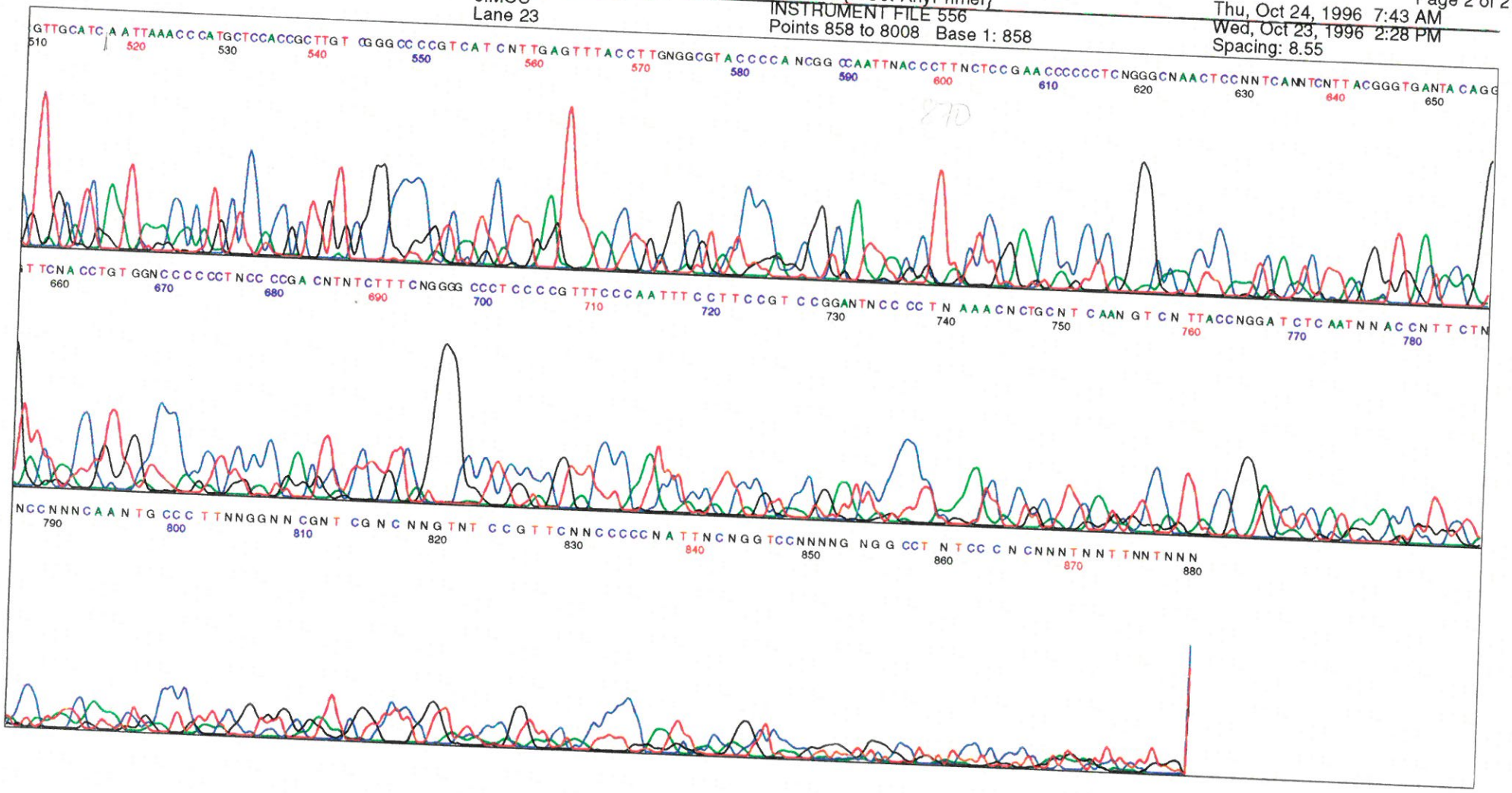
Model 377
Version 2.1.1

23•6.MOS.1

6.MOS
Lane 23

Signal G:285 A:430 T:176 C:189
DT4%Ac{A Set-AnyPrimer}
INSTRUMENT FILE 556
Points 858 to 8008 Base 1: 858

Thu, Oct 24, 1996 7:43 AM
Wed, Oct 23, 1996 2:28 PM
Spacing: 8.55



APPENDIX H (iii): ELECTROPHEROGRAM FOR CLONE KH 34

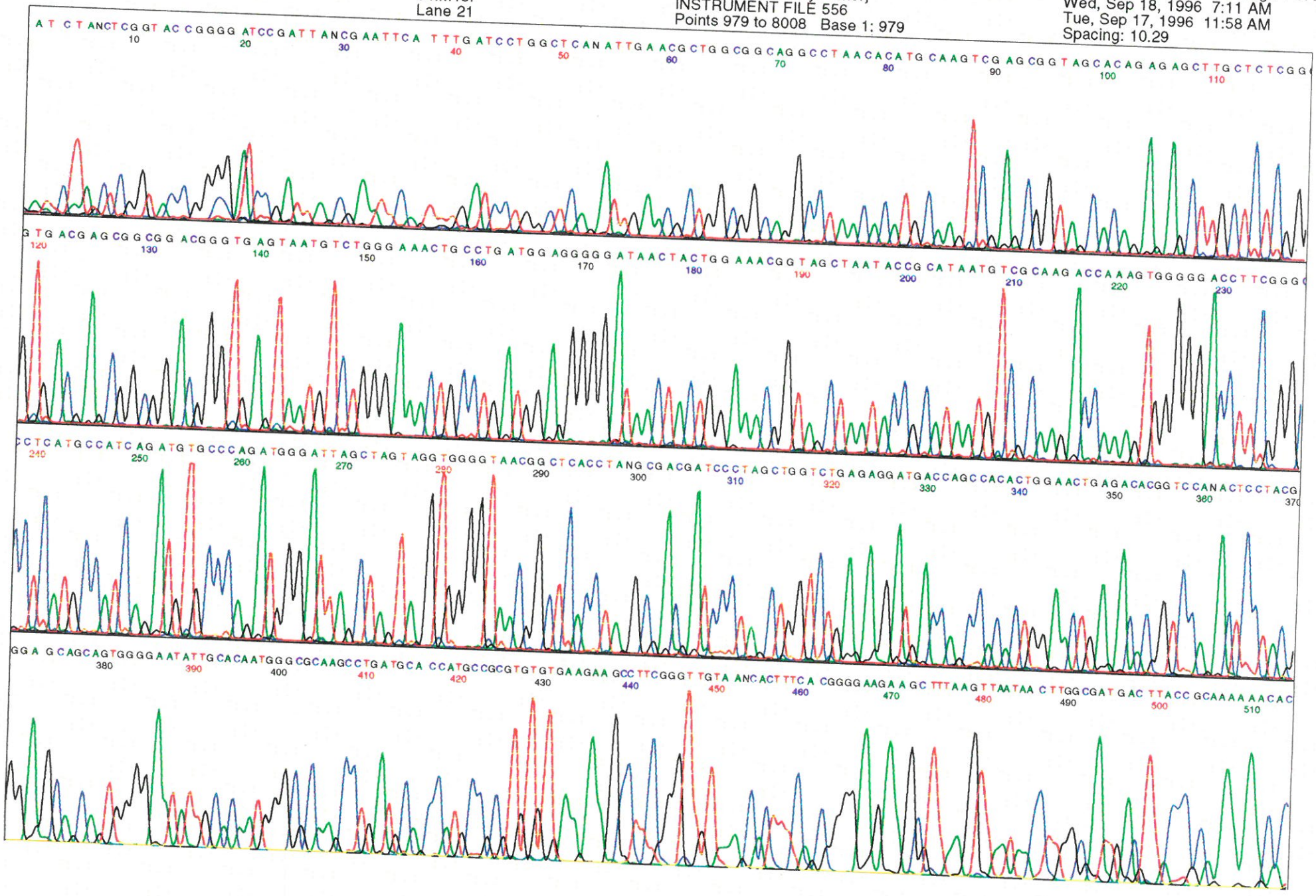
Model 377
Version 2.1.1

21•34.M13F.1

34.M13F
Lane 21

Signal G:258 A:638 T:404 C:342
DT4%Ac{A Set-AnyPrimer}
INSTRUMENT FILE 556
Points 979 to 8008 Base 1: 979

Wed, Sep 18, 1996 7:11 AM
Tue, Sep 17, 1996 11:58 AM
Spacing: 10.29



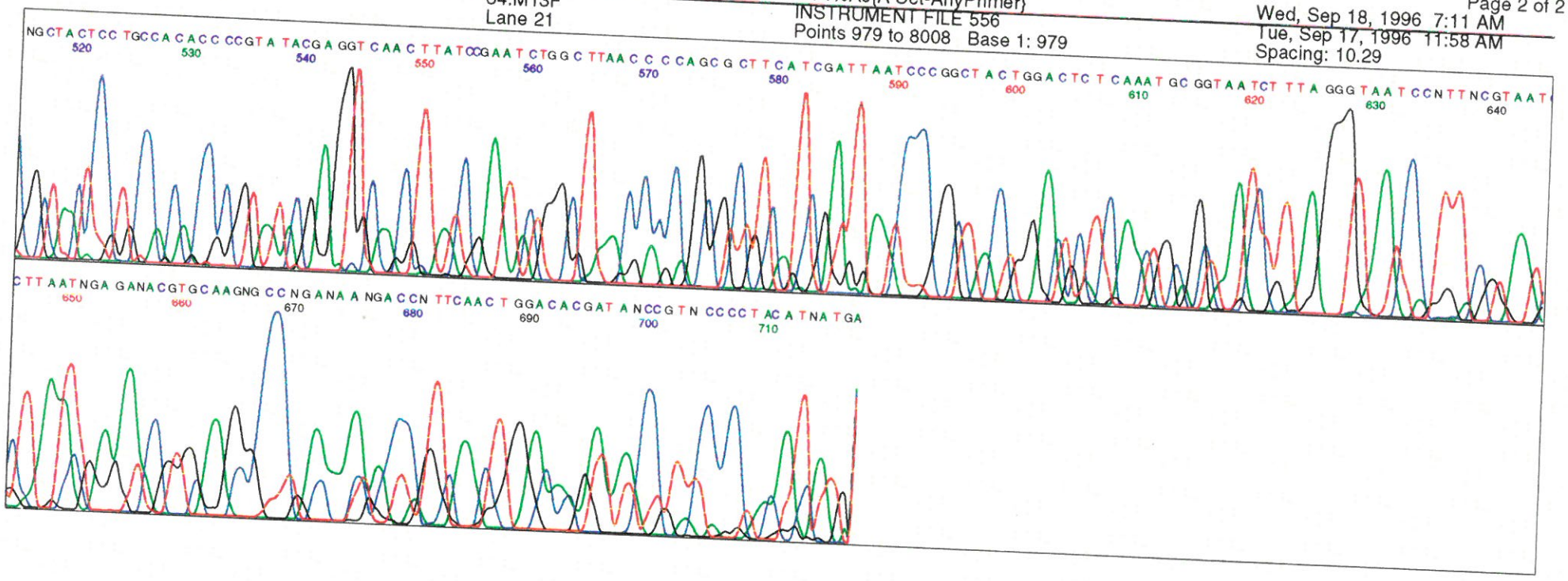
Model 377
Version 2.1.1

21•34.M13F.1

34.M13F
Lane 21

Signal G:258 A:638 T:404 C:342
DT4%Ac{A Set-AnyPrimer}
INSTRUMENT FILE 556
Points 979 to 8008 Base 1: 979

Wed, Sep 18, 1996 7:11 AM
Tue, Sep 17, 1996 11:58 AM
Spacing: 10.29



Applied Biosystems

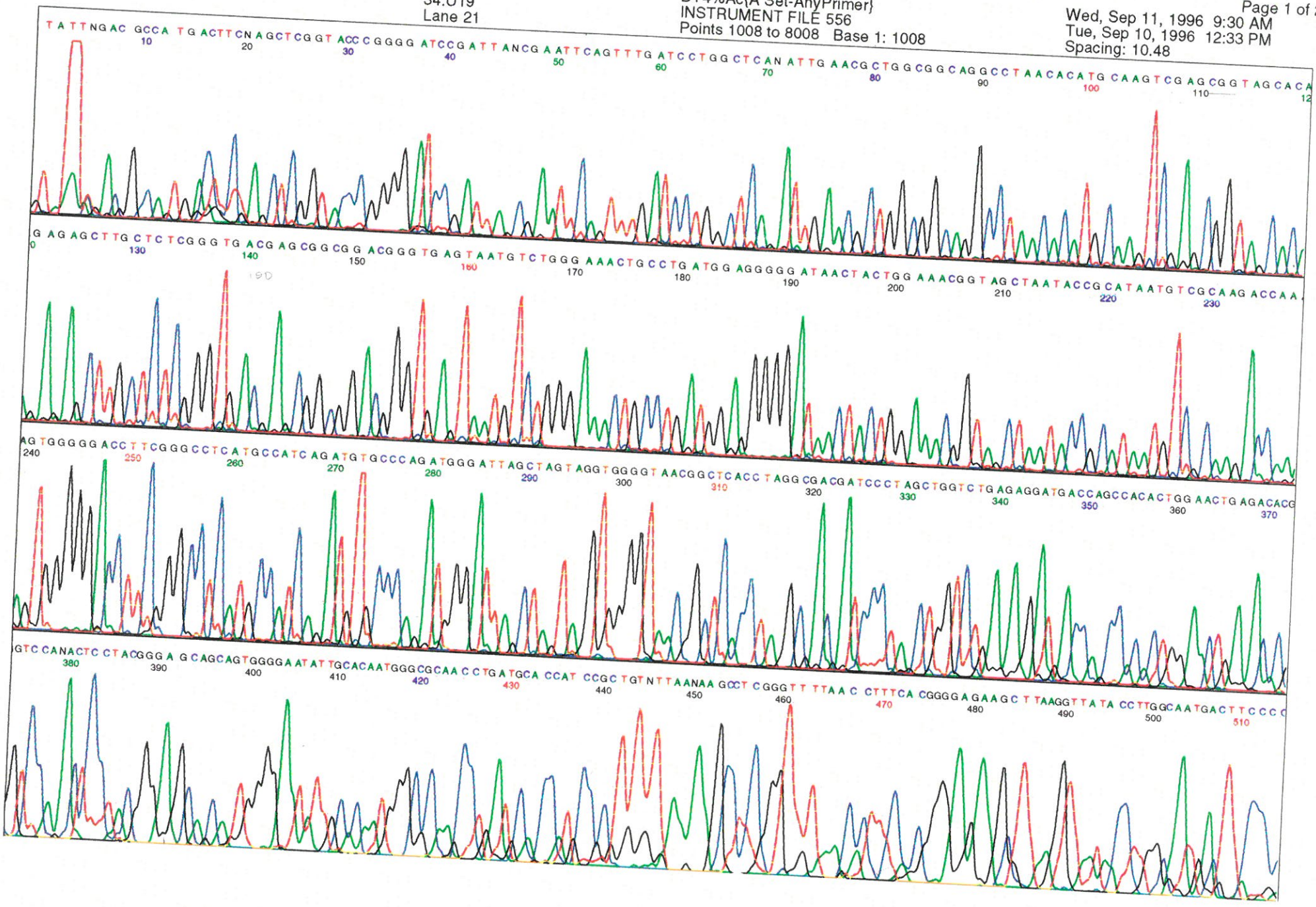
Model 377
Version 2.1.1

21•34.U19.1

34.U19
Lane 21

Signal G:226 A:552 T:290 C:255
DT4%Ac{A Set-AnyPrimer}
INSTRUMENT FILE 556
Points 1008 to 8008 Base 1: 1008

Wed, Sep 11, 1996 9:30 AM
Tue, Sep 10, 1996 12:33 PM
Spacing: 10.48



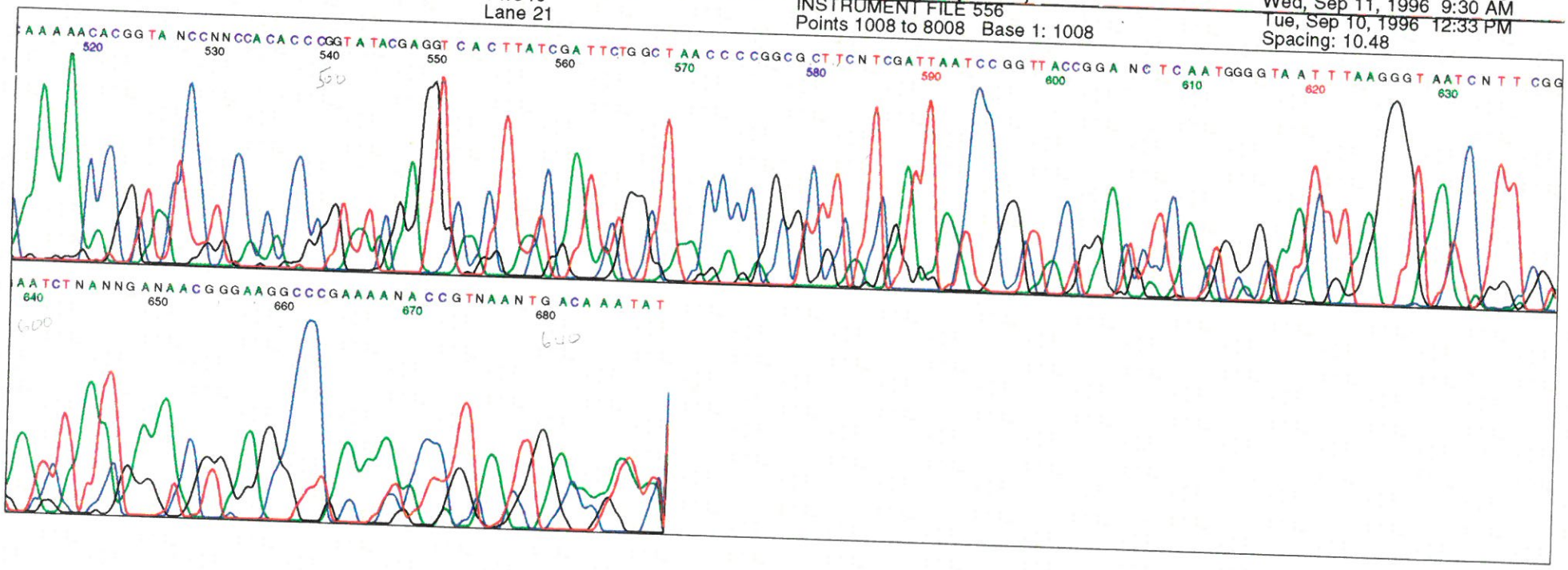
Model 377
Version 2.1.1

21•34.U19.1

34.U19
Lane 21

Signal G:226 A:552 T:290 C:255
DT4%Ac{A Set-AnyPrimer}
INSTRUMENT FILE 556
Points 1008 to 8008 Base 1: 1008

Wed, Sep 11, 1996 9:30 AM
Tue, Sep 10, 1996 12:33 PM
Spacing: 10.48



Applied Biosystems

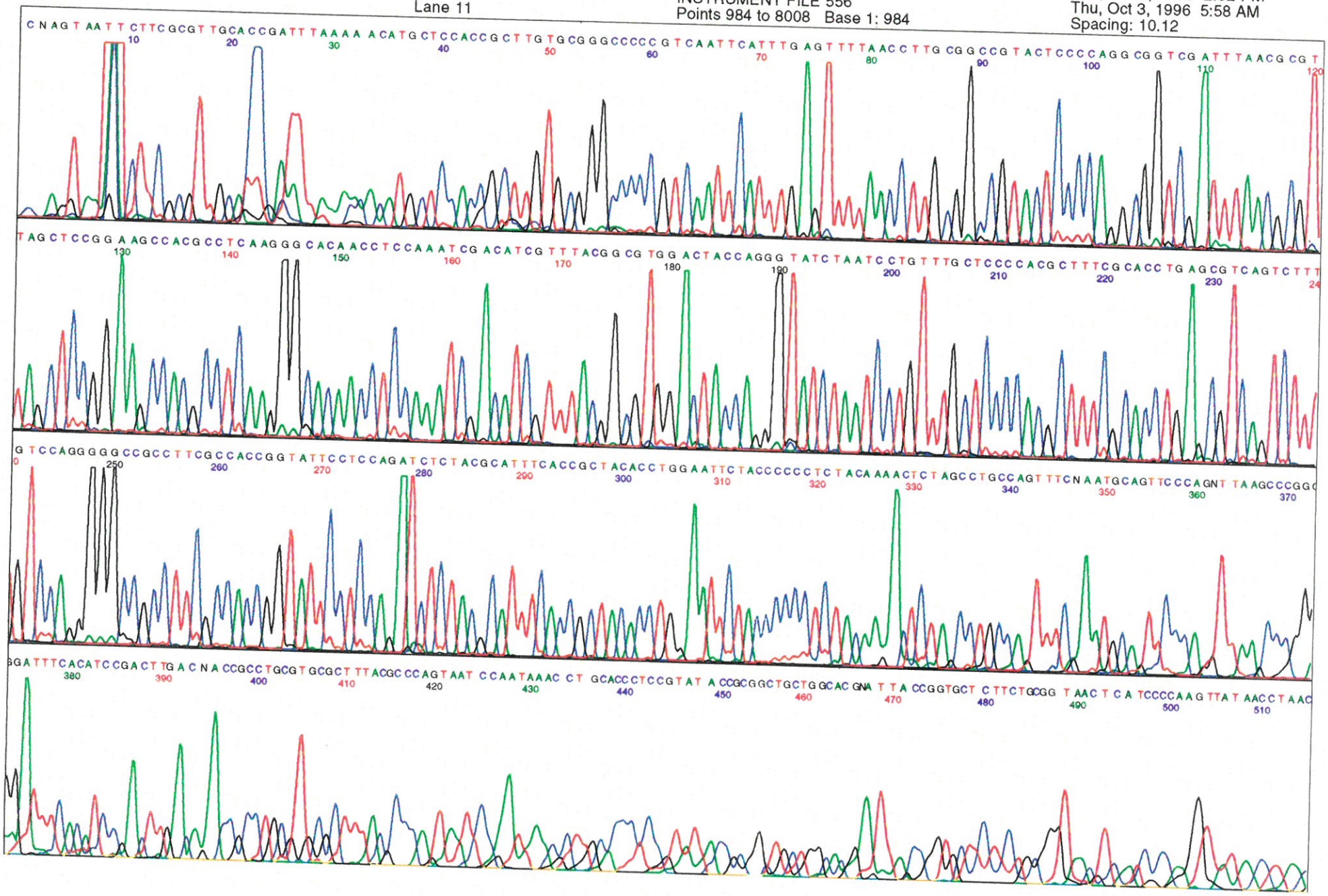
Model 377
Version 2.1.1

11•34.61.1

34.61
Lane 11

Signal G:251 A:597 T:432 C:452
DT4%Ac{A Set-AnyPrimer}
INSTRUMENT FILE 556
Points 984 to 8008 Base 1: 984

Thu, Oct 3, 1996 2:52 PM
Thu, Oct 3, 1996 5:58 AM
Spacing: 10.12



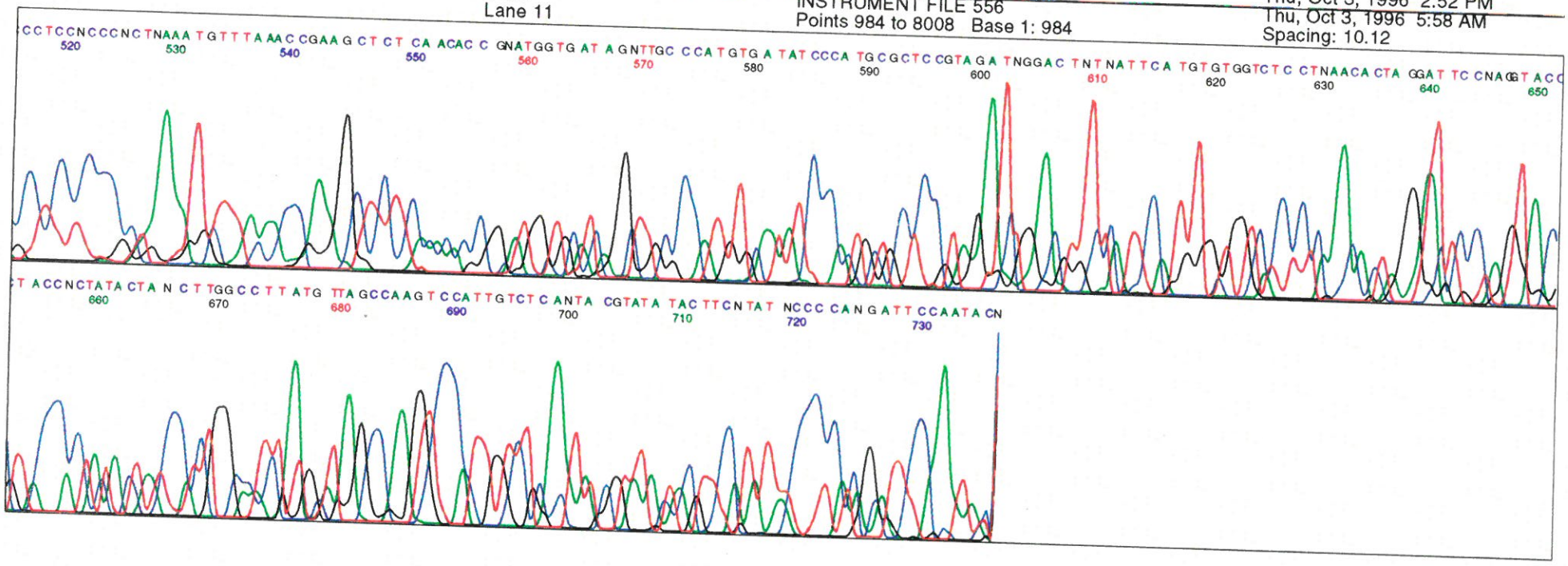
Model 377
Version 2.1.1

11•34.61.1

34.61
Lane 11

Signal G:251 A:597 T:432 C:452
DT4%Ac{A Set-AnyPrimer}
INSTRUMENT FILE 556
Points 984 to 8008 Base 1: 984

Thu, Oct 3, 1996 2:52 PM
Thu, Oct 3, 1996 5:58 AM
Spacing: 10.12



Applied Biosystems

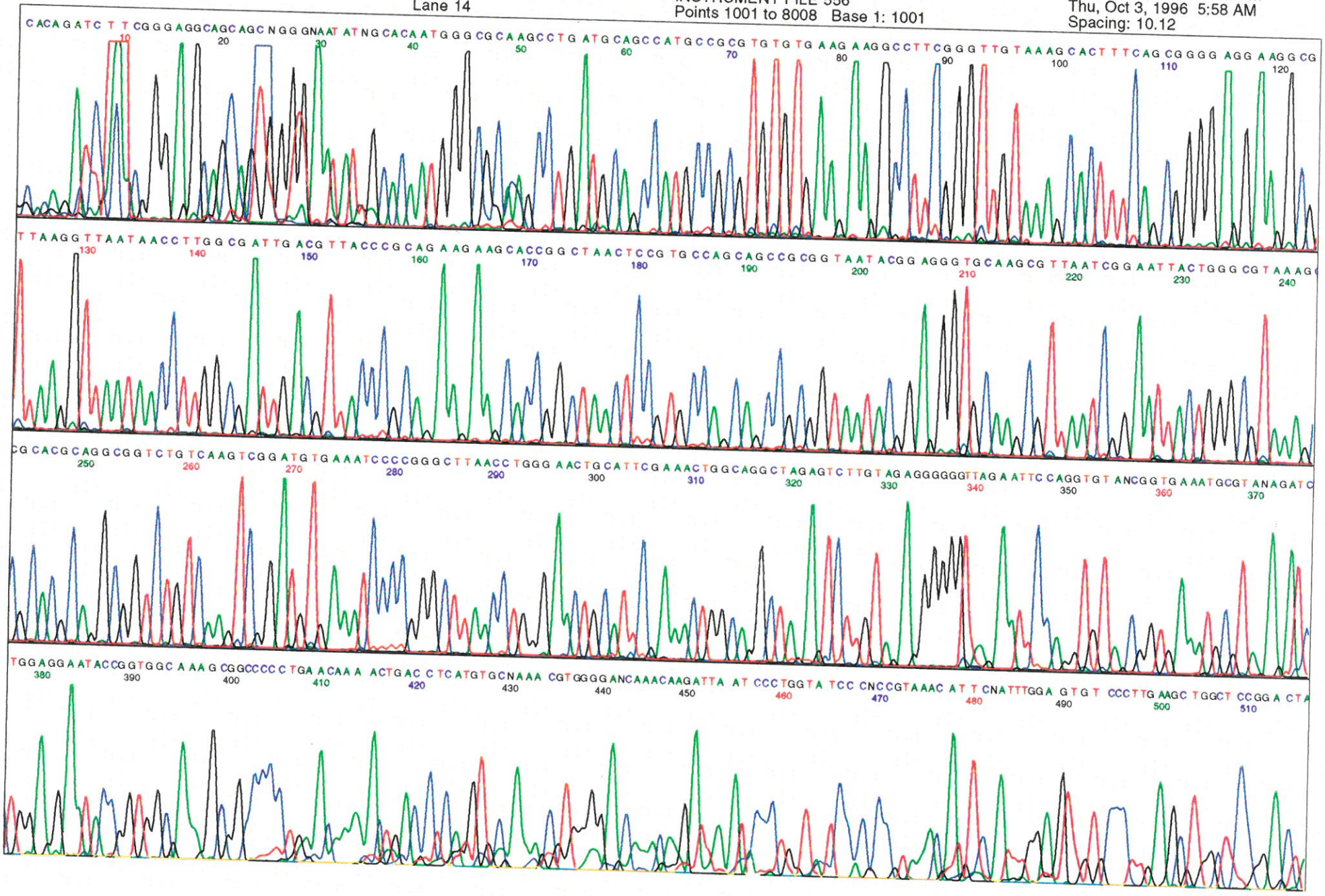
Model 377
Version 2.1.1

14•34.62.1

34.62
Lane 14

Signal G:147 A:377 T:266 C:197
DT4%Ac{A Set-AnyPrimer}
INSTRUMENT FILE 556
Points 1001 to 8008 Base 1: 1001

Thu, Oct 3, 1996 2:52 PM
Thu, Oct 3, 1996 5:58 AM
Spacing: 10.12



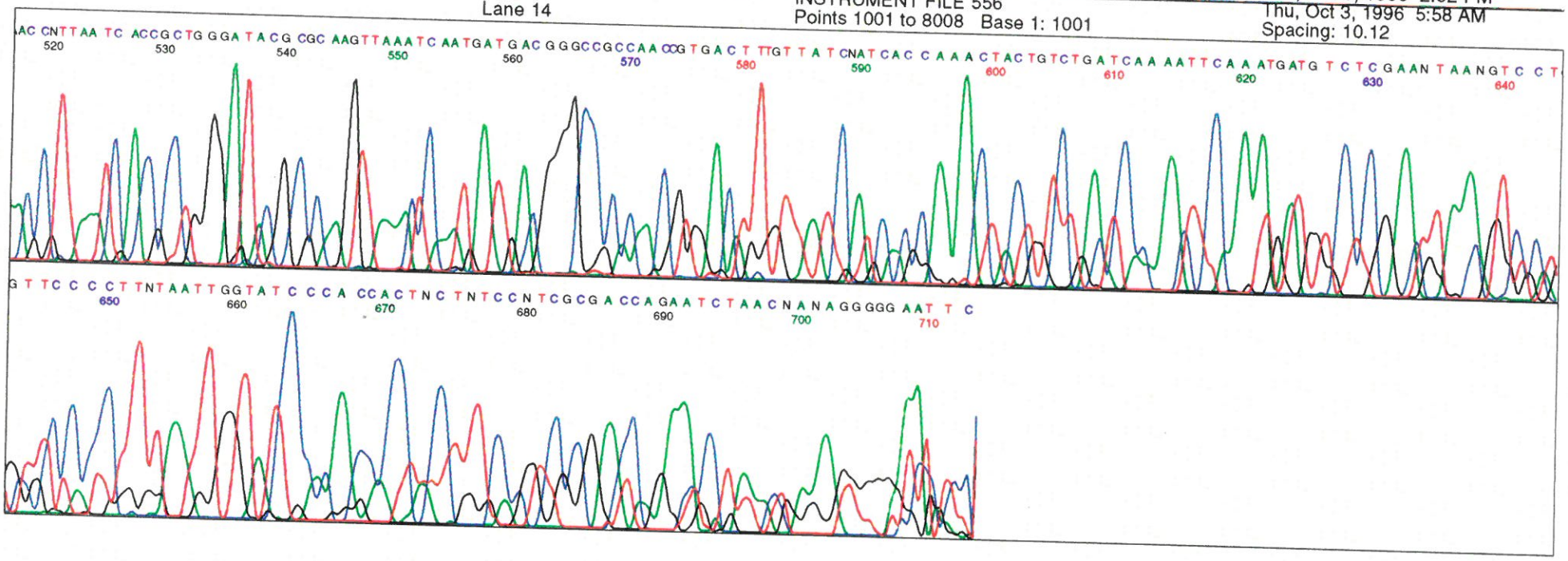
Model 377
Version 2.1.1

14•34.62.1

34.62
Lane 14

Signal G:147 A:377 T:266 C:197
DT4%Ac{A Set-AnyPrimer}
INSTRUMENT FILE 556
Points 1001 to 8008 Base 1: 1001

Thu, Oct 3, 1996 2:52 PM
Thu, Oct 3, 1996 5:58 AM
Spacing: 10.12



Applied Biosystems

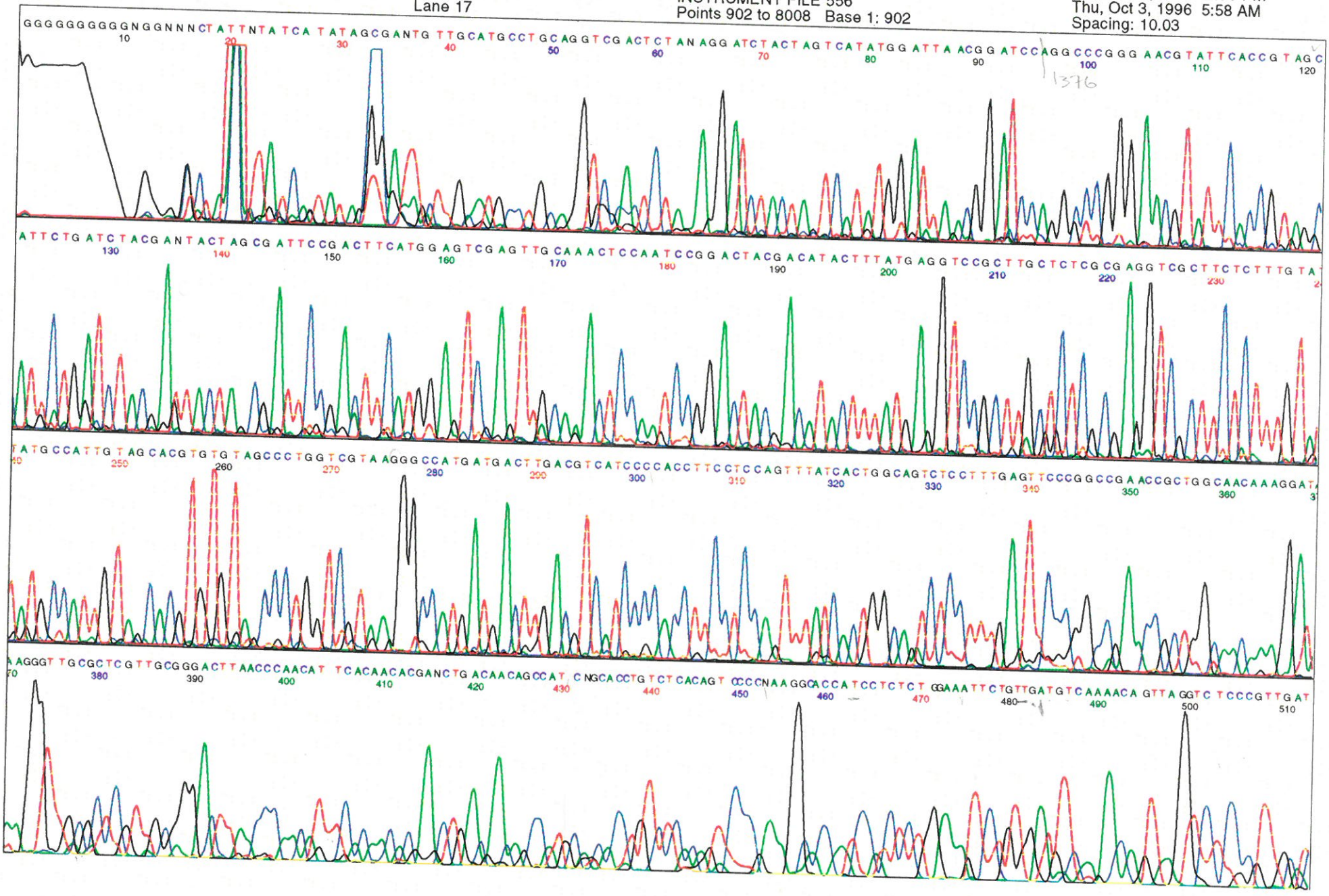
Model 377
Version 2.1.1

17•34.MOS1.1 Reverse

34.MOS1
Lane 17

Signal G:281 A:588 T:461 C:415
DT4%Ac{A Set-AnyPrimer}
INSTRUMENT FILE 556
Points 902 to 8008 Base 1: 902

Thu, Oct 3, 1996 2:53 PM
Thu, Oct 3, 1996 5:58 AM
Spacing: 10.03



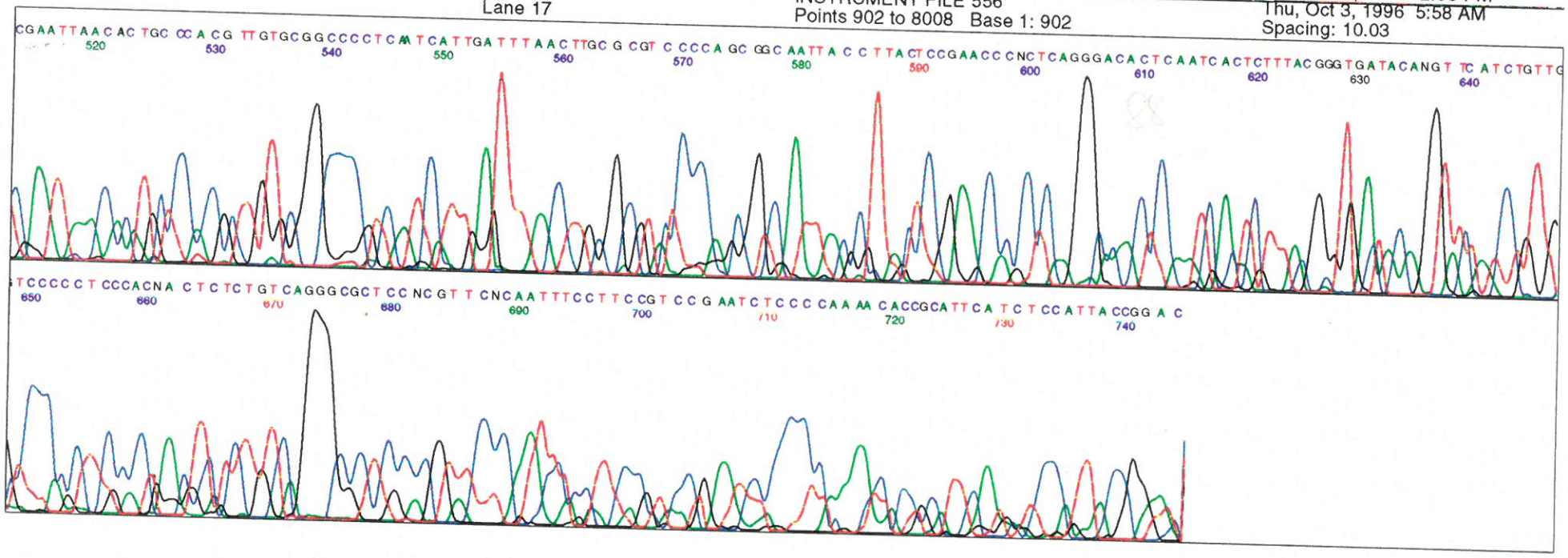
Model 377
Version 2.1.1

17•34.MOS1.1

34.MOS1
Lane 17

Signal G:281 A:588 T:461 C:415
DT4%Ac{A Set-AnyPrimer}
INSTRUMENT FILE 556
Points 902 to 8008 Base 1: 902

Thu, Oct 3, 1996 2:53 PM
Thu, Oct 3, 1996 5:58 AM
Spacing: 10.03



APPENDIX I: DNA SEQUENCE HOMOLOGY DATA

File1: 22.SEQ
Mode: Normal 1 - 1373
File2: 6.SEQ
Mode: Normal 1 - 1373
Matching Percentage (Total Window: 99%, Alignment Window: 99%)

```
   1  AGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCA      50
      ||||||||||||||||||||||||||||||||||||||||||||||||||
   1  AGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCA      50

  51  AGTCGAGCGGTAGCACAGAGAGCTTGCTCTCGGGTGACGAGCGGCGGACG     100
      ||||||||||||||||||||||||||||||||||||||||||||||||||
  51  AGTCGAGCGGTAGCACAGAGAGCTTGCTCTCGGGTGACGAGCGGCGGACG     100

 101  GGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAA     150
      ||||||||||||||||||||||||||||||||||||||||||||||||||
 101  GGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAA     150

 151  ACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGTGGGGGACCTTCG     200
      |||||||||||||||||||| ||||||||||||||||||||||||||||
 151  ACGGTAGCTAATACCGCATAATGTCGCAAGACCAAAGTGGGGGACCTTCG     200

 201  GGCCTCATGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGT     250
      ||||||||||||||||||||||||||||||||||||||||||||||||||
 201  GGCCTCATGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGT     250

 251  AACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGC     300
      ||||||||||||||||||||||||||||||||||||||||||||||||||
 251  AACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGC     300

 301  CACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGG     350
      |||||||||||||||||||||||||||||||||||| ||||||||||
 301  CACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAG-CAGCAGTGGG     350

 351  GAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGA     400
      ||||||||||||||||||||||||||||||||||||||||||||||||||
 351  GAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGA     400

 401  AGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGGGGAGGAAGGCGATAAG     450
      |||||||||||||||||||||||||||||||||||||||||||| ||||
 401  AGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGGGGAGGAAGGCGTTAAG     450

 451  GTTAATAACCTTGGCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAAC     500
      ||||||||||||||||||||||||||||||||||||||||||||||||||
 451  GTTAATAACCTTGGCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAAC     500

 501  TCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAAT     550
      ||||||||||||||||||||||||||||||||||||||||||||||||||
 501  TCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAAT     550

 551  TACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATC     600
      ||||||||||||||||||||||||||||||||||||||||||||||||||
 551  TACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATC     600

 601  CCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTG     650
      |||||||| ||||||||||||||||||||||||||||||||||||||||||
 601  CCCGGGCTTAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTG     650

 651  TAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGG     700
      ||||||||||||||||||||||||||||||||||||||||||||||||||
 651  TAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGG     700
```

```
701  AGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGG      750
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
701  AGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGG      750

751  TGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCC      800
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
751  TGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCC      800

801  GTAAACGATGTCGATTTGGAGGTTGTGCCCTTGAGGTGTGGCTTCCGGAG      850
     |||||||||||||||||||||||||||||||||| ||||||||||||
801  GTAAACGATGTCGATTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAG      850

851  CTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACT      900
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
851  CTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACT      900

901  CAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTT-AATT      950
     |||||||||||||||||||||||||||||||||||| |||| ||||
901  CAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATG-GGTTTAATT      950

951  CGATGCAACGGGAGAC-TTACTGTTTTGACATCCACAGAATTTCCAGAGA     1000
     ||||||||||||||||| ||||||||||||||||||||||||||||||||
951  CGATGCAACGGGAGACCTTACTGTTTTGACATCCACAGAATTTCCAGAGA     1000

1001 TGGATTGGTGCCTTCGGGAACTGTGAGACAGGTGATGCATGGCTGTCGTC     1050
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
1001 TGGATTGGTGCCTTCGGGAACTGTGAGACAGGTGATGCATGGCTGTCGTC     1050

1051 AGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTT     1100
     |||||||||||||||||||||||||||||||||||||||||||||||| ||
1051 AGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACC-TT     1100

1101 ATCCTTTGTTGCCAGCGGTTCGGCCGGGAACTCAAAGGAGATTGCCAGTG     1150
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
1101 ATCCTTTGTTGCCAGCGGTTCGGCCGGGAACTCAAAGGAGATTGCCAGTG     1150

1151 ATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGA     1200
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
1151 ATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGA     1200

1201 CCAGGGCTACACACGTGCTACAATGGCATATACAAAGAGAAGCGACCTCG     1250
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
1201 CCAGGGCTACACACGTGCTACAATGGCATATACAAAGAGAAGCGACCTCG     1250

1251 CGAGAGCAAGCGGACCTCATAAAGTATGTCGTAGTCCGAATTGGAGTTTG     1300
     |||||||||||||||||||||||||||||||||||| ||||||||||||
1251 CGAGAGCAAGCGGACCTCATAAAGTATGTCGTAGTCCGGATTGGAGTTTG     1300

1301 CAACTCGACTCCATGAAGTCGGAATCGCTAGTACTCGTAGATCAGAATGC     1350
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
1301 CAACTCGACTCCATGAAGTCGGAATCGCTAGTACTCGTAGATCAGAATGC     1350

1351 CTACGGTGAATACGTT-CCCGGGCCT.....                      1400
     ||||||||||||||||| |||||||||
1351 CTACGGTGAATACGTTTCCCGGGCCT.....                      1400
```

File1: 22.SEQ
Mode: Normal 1 - 1373
File2: 34.SEQ
Mode: Normal 1 - 1369
Matching Percentage (Total Window: 98%, Alignment Window: 98%)

```
   1  AGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCA      50
      ||||||||||||||||||||||||||||||||||||||||||||||||||
   1  AGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCA      50

  51  AGTCGAGCGGTAGCACAGAGAGCTTGCTCTCGGGTGACGAGCGGCGGACG     100
      ||||||||||||||||||||||||||||||||||||||||||||||||||
  51  AGTCGAGCGGTAGCACAGAGAGCTTGCTCTCGGGTGACGAGCGGCGGACG     100

 101  GGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAA     150
      |||||||||.||||||||||||||||||||||||||||||||||||||||
 101  GGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAA     150

 151  ACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGTGGGGGACCTTCG     200
      |||||||||||||||||||| ||||||||||||||||||||||||||||
 151  ACGGTAGCTAATACCGCATAATGTCGCAAGACCAAAGTGGGGGACCTTCG     200

 201  GGCCTCATGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGT     250
      ||||||||||||||||||||||||||||||||||||||||||||||||||
 201  GGCCTCATGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGT     250

 251  AACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGC     300
      ||||||||||||||||||||||||||||||||||||||||||||||||||
 251  AACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGC     300

 301  CACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGG     350
      |||||||||||||||||||||||||||||||||||| ||||||||||
 301  CACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAG-CAGCAGTGGG     350

 351  GAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGA     400
      ||||||||||||||||||||||||||||||||||||||||||||||||||
 351  GAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGA     400

 401  AGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGGGGAGGAAGGCGATAAG     450
      |||||||||||||||||||||||||||||||||||||||||||| ||||
 401  AGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGGGGAGGAAGGCGTTAAG     450

 451  GTTAATAACCTTGGCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAAC     500
      ||||||||||||||||||||||||||||||||||||||||||||||||||
 451  GTTAATAACCTTGGCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAAC     500

 501  TCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAAT     550
      ||||||||||||||||||||||||||||||||||||||||||||||||||
 501  TCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAAT     550

 551  TACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATC     600
      ||||||||||||||||||||||||||||||||||||||||||||||||||
 551  TACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATC     600

 601  CCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTG     650
      |||||||| ||||||||||||||||||||||||||||||||||||||||
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APPENDIX J: PUBLICATIONS

Culture of *Calymmatobacterium granulomatis*

Granuloma inguinale has emerged as a significant cause of genital ulcer disease in Durban, South Africa [1, 2]. The organism implicated in the etiology of this infection, *Calymmatobacterium granulomatis,* was cultured in embryonated chick eggs in 1943 [3] and from feces on cell-free medium in 1962 [4]. To date, no further reports have been documented, and no cultures are available from any of the type culture collections. We report the successful isolation of *C. granulomatis* in a monocyte coculture system.

Peripheral blood monocytes from a healthy donor were separated through Histopaque 1077 (Sigma Chemicals, St. Louis). Cells were collected, rinsed in Hanks's balanced salt solution, and suspended in RPMI 1640 supplemented with L-glutamine and 10% autologous serum. The cells were transferred to tract vials containing glass coverslips to which the monocytes were allowed to adhere for 1 hour at 37°C in 5% CO_2 in air. Prior to inoculation with the homogenized tissue specimens, the medium was replaced with fresh supplemented RPMI 1640.

Tissue biopsy specimens were obtained from the genital ulcers of patients who presented with the typical clinical picture of granuloma inguinale; if Donovan bodies were observed on Giemsa-stained smears, the specimens were pretreated with amikacin (10 mg/L) for 2 hours, rinsed with PBS (pH, 7.2), homogenized, and suspended in supplemented RPMI 1640. Monocyte monolayers were inoculated in triplicate with 0.5 mL of the suspension and incubated at 37°C in 5% CO_2 in air. After 48 hours, subcultures onto fresh monolayers were performed. Specimens were also inoculated onto a variety of cell-free media, including those recommended by Goldberg [4] and media that support the growth of *Haemophilus ducreyi.* Identical sets of media were inoculated at the time of each subculture. All cell-free media were incubated at 33°C and 37°C aerobically, anaerobically, and microaerophically for 7 days.

Bacteria were successfully grown in the monocyte coculture from the biopsy specimens of three patients, while no growth was observed on any of the other cell-free media. They were successfully passaged to fresh monocytes several times.

In direct smears of patient specimens, the organisms appeared as short, coccobacillary forms with a halo, suggesting the presence of a capsule. However, after 48 hours of incubation with the mononuclear cells, the organisms appeared to elongate; some of the bacilli divided, and the characteristic bipolar appearance remained (figure 1). On subculture in mononuclear cells, the organisms multiplied and appeared as large, elongated coccobacillary forms with a characteristic "safety-pin" appearance. Transmission electron microscopy showed bacteria with a dense capsular layer surrounding a typical gram-negative cell wall and distinct electron-dense granules in the cytoplasm. The bacteria in the monocyte coculture were viable; this observation was reinforced by the presence of extracellular dividing organisms.

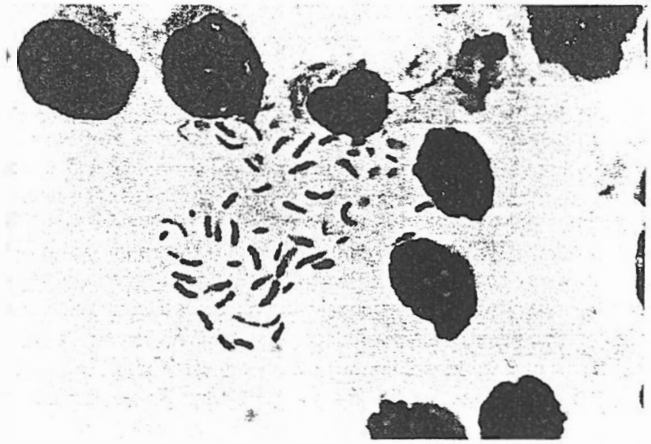

Figure 1. Isolation of *Calymmatobacterium granulomatis* in a monocyte culture (stain, Giemsa; original magnification, ×1,000).

In view of the fact that we were able to demonstrate multiplication of the bacteria in the monocyte coculture system, the absence of growth on a wide variety of cell-free media, and morphological similarities between our isolates and Donovan bodies, our attempt at culturing *C. granulomatis* was successful. This accomplishment paves the way for studies that may lead to the development of molecular-based diagnostic methods; these methods will be more accurate in identifying this organism than is microscopy alone.

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2. Bassa AGH, Hoosen AA, Moodley J, Bramdev A. Granuloma inguinale (donovanosis) in women. An analysis of 61 cases from Durban, South Africa. Sex Transm Dis 1993;20:164–7.
3. Anderson K. The cultivation from granuloma inguinale of a microorganism having the characteristics of Donovan bodies in the yolk sac of chick embryos. Science 1943;97:560–1.
4. Goldberg J. Studies on Granuloma inguinale V. Isolation of a bacterium resembling Donovania granulomatis from the faeces of a patient with granuloma inguinale. Br J Vener Dis 1962;38:99–102.

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J. Med. Microbiol. — Vol. 46 (1997), 579–585

BACTERIAL CHARACTERISATION

Growth and cultural characteristics of *Calymmatobacterium granulomatis* – the aetiological agent of granuloma inguinale (Donovanosis)

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Granuloma inguinale is a chronic destructive granulomatous disease of the genitalia. The clinical diagnosis is often unreliable and the definitive diagnosis is based on the visualisation of 'Donovan bodies' in tissue smears or biopsy specimens. The organism implicated in its aetiology, *Calymmatobacterium granulomatis*, was reported to have been cultured >30 years ago, but little is known about the organism because of its fastidious nature and the difficulty in culturing it. Twenty-two biopsy specimens from female patients with clinical and laboratory-confirmed granuloma inguinale were treated with amikacin 10 mg/L and inoculated in a monocyte co-culture system with peripheral blood mononuclear cells (PBMC) from a single donor and autologous sera. The method was subsequently modified by pretreatment of specimens with vancomycin 5 mg/L and metronidazole 10 mg/L in addition to amikacin 10 mg/L for the purpose of decontamination, pooled blood donor PBMC and by the use of heat-inactivated fetal calf serum instead of autologous serum for culture. This modified method was used to culture additional biopsy specimens and genital ulcer scrapings from female and male patients, respectively. All monocyte co-cultures were examined by a rapid Giemsa (RapiDiff) stain and by an indirect immunofluorescence test with immune sera. Representative cultures were examined by transmission electron microscopy. *C. granulomatis* was successfully isolated in pure culture by the monocyte co-culture system from four biopsy specimens and 14 genital ulcer scrapings. The cultured organisms were visible both intra- and extra-cellularly and were extremely pleomorphic, with characteristic single and bipolar condensation. The numbers of the organisms increased after each passage. All positive cultures showed bright fluorescence when tested with immune sera. Transmission electron microscopy of the cultured bacteria demonstrated a typical gram-negative cell wall consisting of an outer membrane, middle electron opaque layer and an inner plasma membrane. The capsule was thick and electron dense. Numerous electron dense granules were present within the cytoplasm.

Introduction

Granuloma inguinale, also known as Donovanosis, is a chronic granulomatous disease involving the genitalia and surrounding sites. It is found in specific geographical foci, namely New Guinea, north western Australia, south-east India, the Caribbean, parts of South America, parts of central Africa [1] and the KwaZulu/Natal region of South Africa [2].

In 1905, Donovan first described the characteristic intracytoplasmic inclusion bodies in mononuclear cells which were present in smears from oral lesions of a patient who also had ulcerated lesions of the genitalia [3]. These were recognised as the aetiological agent and named *Calymmatobacterium granulomatis* [4]. Currently the laboratory diagnosis of granuloma inguinale relies on the observation of 'Donovan bodies' in tissue smears or biopsy specimens examined by Giemsa and Wright stains [5]. To increase the sensitivity and specificity of the diagnosis, Dieterlies, Warthin-Starry [6] and the rapid Giemsa (RapiDiff) [7] stains have been used.

No real progress has been made with regard to culture of the aetiological agent. In 1943, Anderson [8] reported growth of a gram-negative capsulate bacillus in the yolk of embryonated eggs from a tissue specimen rich in Donovan bodies. Thereafter, between 1943 and 1951, several reports on the primary isolation

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various culture media were inoculated and incubated at appropriate temperatures and atmospheric requirements for a period of 7 days. The coverslip from each vial was air-dried, stained by Gram's and rapid Giemsa (Rapi-Diff) [7] stains and examined by light microscopy at $\times 1000$ magnification. During the development of the culture system, a total of 22 biopsy specimens was subjected to this procedure for co-culture. Pure cultures of *C. granulomatis* were obtained from only three of these specimens and the remaining 19 cultures were overgrown with various micro-organisms, despite amikacin treatment. Twelve of the 19 cultures contained bacteria morphologically resembling *C. granulomatis* among the mixed cultures.

Transmission electron microscopy

Cells from the three positive pure co-cultures were scraped off the coverslips. These suspensions were centrifuged at 12 000 rpm for 15 min and the deposits were fixed in glutaraldehyde 1% in Eagle's minimal essential media with Earle's balanced salts solution (EMEM) (Whittaker M. A. Bioproducts) for 2 h. The specimens were thereafter washed in EMEM and post-fixed in osmium tetroxide 1% in 0.1 M cacodylate buffer for 1 h. The specimens were then dehydrated through ascending grades of alcohol and embedded in araldite epoxy resin. Sections (50 nm) were cut on a Reichert ultracut microtome and stained with Reynold's lead citrate and uranyl acetate before examination with a Joel 100C transmission electron microscope.

Modification of the co-culture method

The co-culture method was modified in three ways: the decontamination procedure for specimens, the source of PBMC and the use of fetal calf serum instead of autologous serum for culture. To establish which antibiotics could be used for specimen decontamination, all contaminating organisms were cultured and tested for their susceptibility to a variety of antimicrobial agents. Thereafter, subsequent biopsy specimens were subjected to a combination of vancomycin (Eli Lilly, USA) 5 mg/L, metronidazole (Rhone-Poulenc) 10 mg/L, and amikacin (Bristol Laboratories) 10 mg/L for 2 h and this combination was found to be most appropriate in eliminating bacterial contamination. As a regular supply of blood from laboratory volunteers is difficult to obtain, PBMC isolated from HIV-negative blood donors attending the Natal Blood Transfusion Services were used. Buffy coat fractions were mixed with pre-warmed unsupplemented RPMI 1640 and transported to the laboratory, and immediately subjected to density gradient centrifugation as described above. The autologous serum was replaced with heat-inactivated fetal calf serum (Delta Bioproducts, South Africa). This modified method was used for co-culturing additional biopsy specimens from female patients and tissue scrapings obtained from patients presenting with genital ulcer disease to the Sexually Transmitted Diseases (STD) Clinic, City Health, Durban.

Culture from tissue scrapings

In a study to determine the aetiology of genital ulcer disease amongst adult males, 200 consecutive patients with genital ulceration were investigated. Tissue scrapings were obtained with a 100 μl plastic bacteriological loop (Bibby Sterilin) and collected in 500 μl of PBS, pH 7.2. One hundred μl were transferred to 500 μl of decontamination medium containing RPMI 1640 supplemented with fetal calf serum 10% and amikacin 10 mg/L, vancomycin 5 mg/L and metronidazole 10 mg/L. After 2 h the tubes were centrifuged at low speed, excess supernate was discarded and the deposit was resuspended in 1 ml of RPMI 1640 supplemented with fetal calf serum 10% without antibiotics, before inoculation on to mononuclear cells.

Immunofluorescence

Slides of all positive co-cultures were incubated for 30 min at 37°C in a humidified chamber with sera from two patients with granuloma inguinale who presented with lesions of 3 months and 1 year duration. Serum from a volunteer with no past or present history of genital ulcer disease was also tested. All sera were tested at dilutions of 1 in 80 and 1 in 160. The slides were washed and re-incubated with fluorescein isothiocyanate-labelled mouse anti-human IgG (Wellcome Diagnostics) for 30 min. Smears obtained from the ulcerative lesions of patients with granuloma inguinale which showed characteristic 'Donovan bodies' were used as positive controls. Cultures of laboratory strains of *Klebsiella pneumoniae*, *K. oxytoca*, *K. aerogenes*, *Enterobacter cloacae*, *Citrobacter freundii* and *Escherichia coli* were used as negative controls. Both positive and negative control slides were tested with the patients' sera and serum from the volunteer.

Results

During development of the co-culture system, pure cultures of *C. granulomatis* were obtained from three of 22 biopsy specimens from women with granuloma inguinale (Donovanosis). An additional biopsy specimen yielded pure growth after use of the modified method. Fourteen of the 200 tissue scrapings from men presenting with genital ulcers to the STD Clinic yielded growth in the monocyte co-culture after incubation for 48 h. None of these grew on any of the cell-free culture media (Table 1) that were incubated for 7 days.

All co-cultures, i.e., the four from biopsy specimens and 14 from tissue scrapings, tested positive in the indirect immunofluorescence test with the patients' sera (Fig. 1A) and negative with the serum from the volunteer at both dilutions. Direct smears from lesions

The extracellular bacteria were in close proximity to the monocytes. The staining reaction revealed characteristic single and bipolar condensation as described for 'Donovan bodies' (Fig. 2A). The bacteria were pleomorphic with either bulging or tapered ends. Dividing bacilli were evident. The size range was 0.5–2.0 μm in width and 1.0–2.5 μm in length. After subsequent passages an increase in the number of bacterial cells was observed with a number of coccobacillary forms present (Fig. 2B). Around all the cells a translucent area was present, suggestive of capsule formation. The single and bipolar condensation was still evident. Gram's stain of the cultures showed that the bacteria were gram-negative (Fig. 2C), although they stained poorly.

On transmission electron microscopy, bacteria appeared singly or in clusters (Fig. 3A) with a number of extracellular dividing organisms (Fig. 3B). They exhibited a characteristic gram-negative cell wall (Fig. 3C), consisting of an outer membrane (OM), a middle electron opaque layer (MW) and an inner plasma membrane (IM). The periplasmic space (PS) between the outer and inner membrane was electron lucent. Both plasma and outer membranes were approximately 7.5 nm thick, each displaying the typical trilaminar nature. The capsule (G) was thick, dense and fuzzy (Fig. 3D). A membrane-like structure (TM) was observed on the periphery of the capsule. The cytoplasm was rich in ribosomes, many of them occurring in aggregates. Electron-dense granules (DG) were prominent (Fig. 3D). Pili (fimbriae) or flagella were not identifiable.

Discussion

The resurgence of granuloma inguinale in the KwaZulu/Natal region of South Africa prompted this attempt to culture the aetiological agent, *C. granulomatis*. Initial efforts to emulate the work of the early researchers (1940–1960) met with no success. This is also in keeping with the unavailability of the organism from any international type culture collection. However, when the yolk sacs of 5–7-day-old fertile chick eggs were used, the aetiological organism was maintained but could not be subcultured for growth.

The choice of PBMC for culture was based on the visualisation of the causative agent within mononuclear cells of specimens (smears and biopsy) of infected tissue. Such a system allows human monocytes and macrophages to control the growth of microbial pathogens and provides an opportunity to study host–parasite interactions [18]. During the initial phase of the study, PBMC were obtained from a

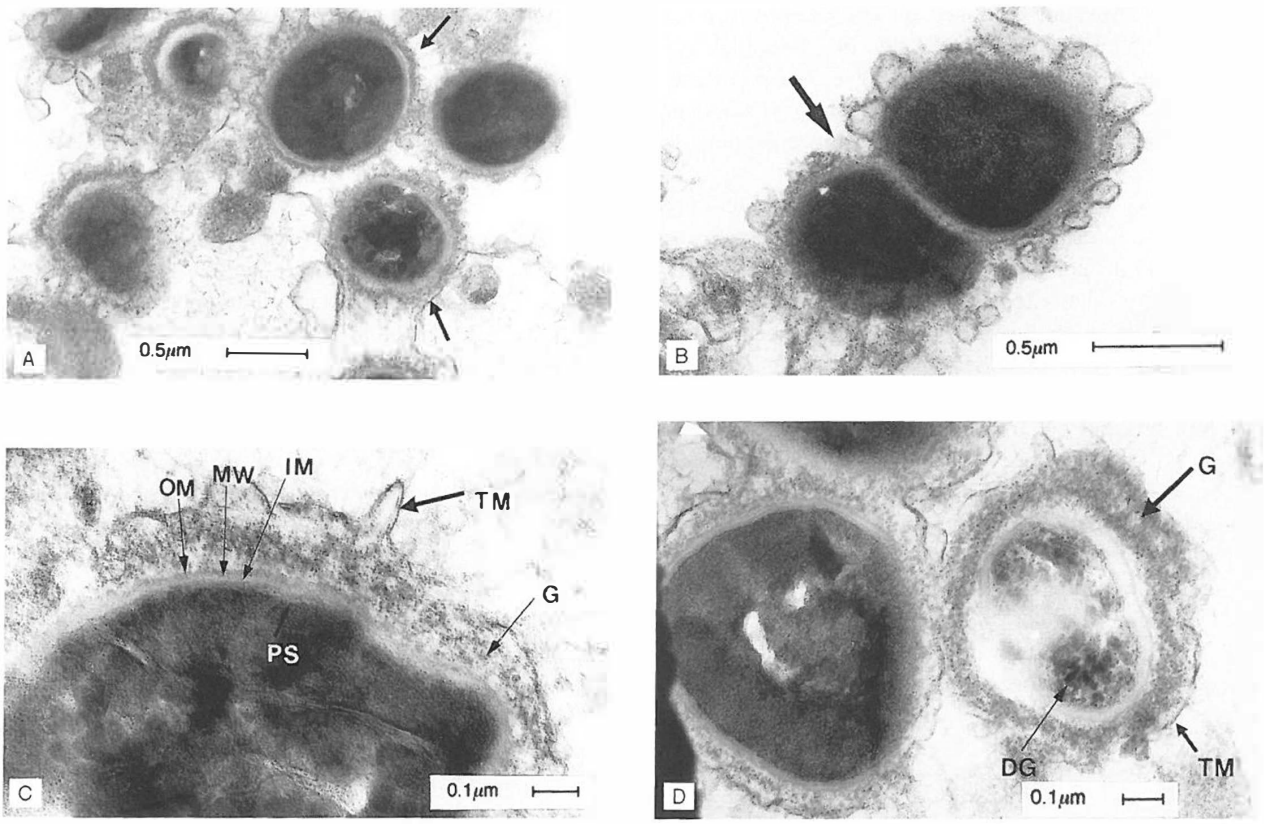

Fig. 3. Electron microscopic characteristics of *C. granulomatis* from monocyte co-cultures. **A**, Large number of extracellular organisms. **B**, Dividing extracellular organisms. **C**, Gram-negative cell wall structure of the organism consisting of an outer membrane (OM), middle electron opaque layer (MW), inner plasma membrane (IM) with periplasmic space (PS) and evidence of a trilaminar membrane (TM) around the capsule. **D**, Characteristic thick, fuzzy and electron dense capsule (G) with numerous electron dense granules (DG) within the cytoplasm.

electron lucent areas are in reality intracellular vacuoles in which the bacteria are situated and not bacterial capsules. In the present study, electron microscopic features are described from cultured specimens and not from biopsy specimens. Davis *et al.* [24] suggested that the capsule is unusual as it is large in size and has a capsular membrane. Whether the membrane-like structure observed on the periphery of the capsule in this study represents the capsular membrane seen in Davis' study is unclear. Clearly there was no evidence of pili (fimbriae) or flagella as suggested by Chandra *et al.* [28]. The bacterial cell wall was characteristically that of a gram-negative bacterium as desribed by others [24, 26].

This study developed a culture method for *C. granulomatis* which can be applied to routine clinical specimens such as ulcer scrapings, as well as biopsy samples of large lesions. The successful culture of the aetiological agent of granuloma inguinale will enable the collection of a sufficient number of bacterial strains to study the biological characteristics and virulence attributes of this micro-organism, to determine its antimicrobial susceptibility profile and to develop a definitive diagnostic test. This will lead to a better understanding of the epidemiology and pathogenesis of this disease.

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ULTRASTRUCTURAL STUDY OF *CALYMMATOBACTERIUM GRANULOMATIS*: THE CAUSATIVE AGENT OF GRANULOMA INGUINALE

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Granuloma inguinale is a sexually transmitted disease characterised by chronic granulomatous lesions of the genitalia and surrounding sites. The causative agent, *Calymmatobacterium granulomatis*, has not been cultured in bacteriological cell free media which has hindered its characterisation. Although the electron microscopic appearance of *C. granulomatis* in tissue specimens has been described, it is not specific. The successful isolation of *C. granulomatis* in a monocyte co-culture system[1] enabled us to study the ultrastructural characteristics of this organism and make comparisons to bacteria seen in tissue biopsy specimens.

Tissue biopsy specimens from two patients with granuloma inguinale, diagnosed by visualisation of "Donovan bodies" in smears, were fixed in Karnovsky's fixative. Cells from monocyte co-cultures were scraped off glass coverslips, centrifuged at 12 000g for fifteen minutes and the deposits fixed in 1% glutaraldehyde in Eagles minimal essential media with Earle's balanced salts solution for two hours. All specimens were processed, embedded and sectioned for transmission electron microscopy using conventional techniques.

On transmission electron microscopy bacteria were present both singly and in clusters in both the cultured specimen and the biopsies. The cell wall consisted of an outer membrane (OM), a middle electron opaque layer (ML) and an inner plasma membrane (IM). The periplasmic space (PS) between the outer and inner membrane was electron lucent. Both IM and OM were approximately 7.5nm thick and displayed the typical trilaminar nature. These features are consistent with those found in Gram negative organisms and have been described by other workers[2,3,4]. The capsule (C) was thick, electron dense and fuzzy, but this description appears to be discrepant in relation to other studies where it has been described to be an electron lucent area around the organism[2,3]. Capsule characteristics have been described extensively for other bacterial organisms, having a similar electron dense character and this variation in the configuration of the capsules may reflect the organisms virulence. Pili or flagella were not identifiable as described in another study[4]. There was no evidence of particles either within or on the organism to suggest the presence of empty phage heads or bacteriophage indicating that *C. granulomatis* may be a phage modified bacterium[3].

This study confirms the Gram negative nature of the cell wall of *C. granulomatis*. The ultrastructure of local isolates showed a unique electron dense capsule and the absence of bacteriophage, phage particles, pili or flagella.

The financial support of the Medical Research Council and the University of Natal is gratefully acknowledged.

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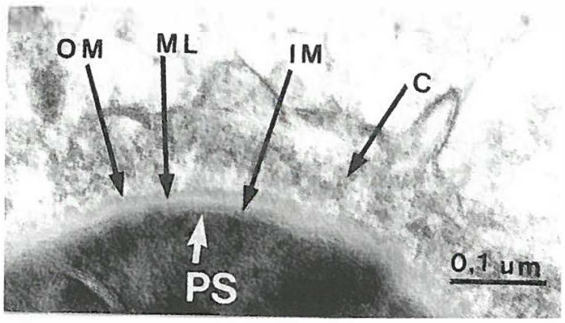

Fig. 1. Gram negative cell wall structure of *C. granulomatis*. Outer membrane (OM), middle electron opaque layer (ML), inner plasma membrane (IM), periplasmic space (PS), capsule (C).

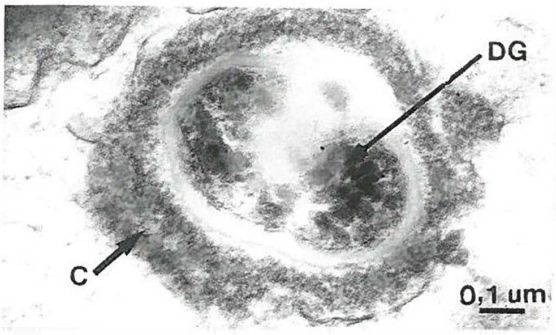

Fig. 2. Electron-dense granules (DG), capsule (C).

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ULTRASTRUCTURAL MORPHOLOGY OF *CALYMMATOBACTERIUM GRANULOMATIS* : THE AETIOLOGICAL AGENT OF GRANULOMA INGUINALE

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Granuloma inguinale is a sexually transmitted disease characterised by chronic granulomatous lesions of the genitalia and surrounding sites. The aetiological agent, *Calymmatobacterium granulomatis*, has not been cultured in cell free media and only recently cultured in monocytes [1]. The lack of a suitable culture has hindered its characterisation with the ultrastrustural characteristics being non-specific [2-4].

1. Aim

This study was undertaken to determine the ultrastructural morphological characteristics of *C. granulomatis* in culture and in tissue biopsy specimens.

2. Methods

Tissue biopsy specimens from patients with granuloma inguinale, diagnosed by visualisation of "Donovan bodies" in smears, were fixed in Karnovsky's fixative. Cells from monocyte co-cultures were scraped off glass coverslips, centrifuged at 12 000g for fifteen minutes and the deposits fixed in 1% glutaraldehyde in Eagles minimal essential media with Earle's balanced salts solution for two hours. All specimens were processed, embedded and sectioned for transmission electron microscopy.

3. Results

C. granulomatis were seen extracellularly in culture while in tissues they were located predominantly intracellularly within membrane bound vacuoles in the cytoplasm of the macrophages and occasionally within extracellular spaces. Figure 1 shows *C. granulomatis* in culture with the cytoplasm of the bacteria containing ribosomes and electron dense granules. The capsule consisted of dense homogeneous material of relatively high electron contrast. Figure 2 shows the cell wall structure of the Gram negative organism consisting of an outer membrane, a middle electron opaque layer and an inner plasma membrane. Figure 3 shows a bacillus within a phagocytic vacuole. The filamentous skein of nucleoplasm, electron dense granules, ribosomes and capsule are shown. Capsular material beween adjacent bacilli are seen to be continuous with each other. The fine fibrillar strands are evident attaching the capsule to the limiting membrane. In tissue specimens the homogeneous layer surrounding the bacterial body varied from electron dense to delicate web like structures with varying denseness and thickness. Surface structures such as pili (fimbriae), flagellae and bacteriophage were not identifiable.

4. Conclusions

This study confirms the similarity of the organism in culture and in tissue biopsy specimens, having a Gram negative cell wall structure, electron dense capsules and the absence of bacteriophage particles, pili or flagella.

The financial support of the Medical Research Council and the University of Natal is gratefully acknowledged.

MORPHOLOGY OF *Calymmatobacterium granulomatis* - "DONOVAN BODIES"

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Granuloma inguinale is a chronic granulomatous disease involving the genitalia. The aetiological agent, *Calymmatobacterium granulomatis*, has not been cultured in cell free media and only recently cultured in monocytes[1]. The successful isolation of *C. granulomatis* in a monocyte co-culture system enabled us to study the microscopic morphology of this organism and make comparisons to bacteria seen *in vivo*.

Tissue biopsy specimens were obtained from genital ulcers of three patients with the typical clinical picture of granuloma inguinale. RapiDiff stained smears were prepared and examined for "Donovan bodies". The specimens were pretreated with antimicrobial agents, homogenised and co-cultured with peripheral blood mononuclear cells (PBMNC) in RPMI with 10% foetal calf serum and incubated at 37°C in 5% CO_2 in air. The coverslips from each vials were air dried, stained by RapiDiff and examined using an Olympus BX50 microscope.

From direct smears, the infected macrophages appeared enlarged with the nucleus displaced to the periphery of the cell cytoplasm. The bacteria were found intracellularly in the cytoplasm, either within a single vacuole or multiple vacuoles. These varied from being coccobacillary to bacillary and appeared to be slender with tapering ends or short with an intense staining reaction. Both encapsulated and non-encapsulated forms were present (Fig. 1).

From cultured PBMNC, pleomorphic bacteria were visualised both intra and extracellularly. These varied in length with bulging or tapered ends. The chromatin material was positioned centrally or at the periphery of the intensely stained bacilli. A large number of bacilli appeared to divide into two unequal cells. Subcultures of bacteria displayed morphological features similar to those in primary cultures, but many of the cells were extremely pleomorphic with coccobacillary forms (Fig. 2). In later experiments 5 to 7 day monocytes were used for secondary cultivation. A large number of bacteria were seen intracellularly within vacuoles often displacing the nuclei to the periphery. When the bacilli were seen in clusters, the capsules appeared to coalesce to form a large halo around the bacteria (Fig. 3).

This study demonstrates the pleomorphic nature of the organism, *C. granulomatis*.

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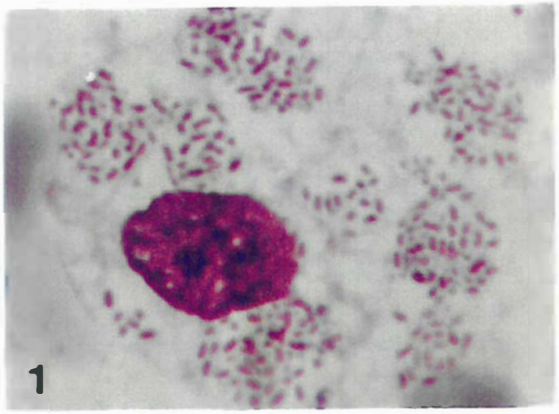

Fig. 1. A large mononuclear cell filled with "Donovan bodies" in the cytoplasm which is vacuolated or in pockets of vacuoles (Mag x1000).

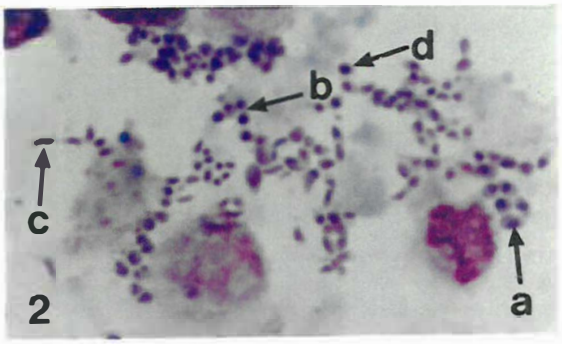

Fig. 2. Monocyte co-cultures displaying extra-cellular pleomorphic bacteria with single (a) and bipolar (b) condensation with tapering ends (c), coccobacillary forms (d) (Mag x1000).

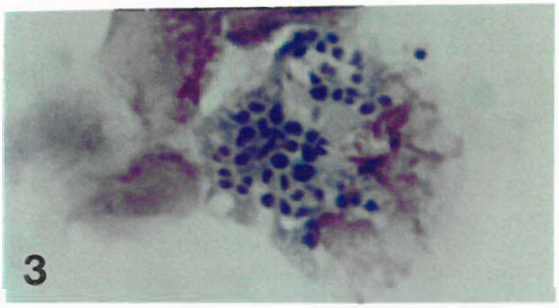

Fig. 3. Intracellular bacteria seen singly or in clusters in vacuoles with displacement of the nucleus (Mag x1000).

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SECTION TITLE

Ultrastructure of *Calymmatobacterium granulomatis*: comparison of culture with tissue biopsy specimens

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The ultrastructural features of cells of *Calymmatobacterium granulomatis* from monocyte co-cultures and tissue biopsy specimens were compared. In cultures the bacteria were mainly extracellular, i.e., not within membrane-bound vacuoles. The bacterial body was surrounded by a uniformly extensive homogeneous layer with a relatively high electron density. This layer varied considerably in tissue biopsy specimens, having either homogeneously electron-dense or delicate web-like structures with varying density and thickness. In tissue specimens the bacteria were located predominantly within vacuoles of varying sizes in the cytoplasm of the macrophages and, occasionally, extracellularly within the intercellular spaces of the stroma. The bacterial cytoplasm contained ribosomes scattered throughout with electron-dense granules located peripherally. The trilaminar cell wall structure was typical of a gram-negative organism, comprising an outer membrane, a middle electron-opaque layer and an inner plasma membrane. Surface structures such as fimbriae, flagella and bacteriophages were not identified in specimens from either source.

Introduction

Calymmatobacterium granulomatis is the aetiological agent of granuloma inguinale (Donovanosis), a chronic granulomatous disease involving the genitalia and surrounding tissues. It has been recognised recently as a significant cause of genital ulcer disease in the KwaZulu/Natal region of South Africa [1–3].

Histological examination of granulation tissue from infected patients reveals the presence of macrophages, plasma cells, polymorphonuclear neutrophils and, occasionally, lymphocytes, multinucleated giant cells and dendritic cells [4–6]. The infected macrophages in the diseased tissue are highly activated, containing a variable number of bacteria within intracellular vacuoles. The observation of these intracellular bacteria is important for the diagnosis of the disease [5, 7].

C. granulomatis is not easily cultivable *in vitro* and, therefore, has been poorly studied. Previous studies on the ultrastructure of the organism have used infected biopsy material fixed by procedures that were only adequate for the preservation of host tissue, but which did not satisfactorily preserve bacterial ultrastructure [8]. The ultrastructural characteristics of *C. granulomatis* has a trilaminar cell wall structure and is considered to be in accordance with its gram-negative staining. The cytoplasm is known to contain electron dense granules [9, 10]. The bacteria have also been described as being surrounded by a clear layer (electron-lucent), the width of which varies considerably [6]. These have been described as capsules, but they appear to differ from those of other bacteria, which are invariably electron-dense [11]. The presence of bacteriophages either attached to the cell wall or as empty phage heads within the organism have been described [4, 7] or have been found to be absent [5, 6, 9]. The presence of pili (fimbriae) and vesicles evaginating from the cell membrane has been reported occasionally [5, 9, 10]. Therefore the ultrastructural characteristics of *C. granulomatis* are not clearly defined. Recently, the growth of *C. granulomatis* was achieved in a monocyte co-culture system and its presence was confirmed morphologically [12].

This study was undertaken to compare the ultrastructural features of *C. granulomatis* in tissue biopsy specimens with those observed in culture.

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