The Prevalence of Members of the "Red Complex" in Pregnant Women as Revealed by PCR and BANA Hydrolysis.

By

Claude BAYINGANA

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Supervisor: Prof. Charlene Africa

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CLAUDE BAYINGANA

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ABSTRACT

Increased levels of oestrogen and progesterone during pregnancy may lead to periodontal disease. The anaerobic Gram-negative bacteria called **red complex** (*Porphyromonas gingivalis*, *Tannerella forsythensis* and *Treponema denticola*) are frequently associated with periodontal disease. Periodontopathogens produce toxins and enzymes which can enter the bloodstream and cross the placenta to harm the foetus. The response of the mother's immune system to infection by these periodontopathogens, brings about the release of inflammatory mediators which may trigger preterm labour or result in low birth-weight infants. The purpose of this study was to examine the prevalence of **red complex**, using BANA and PCR in subginginval plaque samples from pregnant women**.** Subgingival plaque samples were obtained from pregnant women between the ages of 17 to 45 years attending a Mitchells Plain ante-natal clinic. Plaque samples were analyzed by the enzymatic BANA-test for detection of the presence of **red complex** and DNA was extracted and analyzed using 16 rDNA-Polymerase Chain Reaction (PCR).

Seventy-nine percent of pregnant women showed gingival index scores of ≥ 1 of which 74.24% harboured by at least one of the members of the **red complex**. *P.gingivalis* was the most prevalent of the three members of the **red complex**. Findings of this study confirmed a need for dental preventive measures in pregnant women and microbial monitoring of suspected periodontopathogenes. This could be achieved by joint co-operation between Maternity Obstetric Units (MOU), Dentistry and oral microbiology departments. The results of this study revealed that although PCR is more sensitive than BANA in detecting members of the **red complex,** BANA showed a better association with the indices used to diagnose periodontal disease.

DECLARATION

I declare that this work is my own work, that it has not been submitted before for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Claude BAYINGANA **October 2005**

Signed:……..

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Finally, I ask for the forgiveness of those whose contributions to this study, I have inadvertently forgotten.

DEDICATION

I dedicate this thesis to my children Axel, Tricia, Nick and Militia for their love and sacrifice for my success.

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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction

Periodontal disease is an infection of the tissues surrounding and supporting the teeth and may vary from inflammation of the gingivae alone (gingivitis**)**, to severe inflammation of the periodontal ligament (periodontitis**)** as a result of a non-specific proliferation of the normal gingival crevice microflora due to poor oral hygiene (Marsh and Martin, 1992). Plaque bacteria may produce disease directly, by invasion of the tissues, or indirectly via bacterial toxins.

In the early stage of periodontal disease, the gingivae become red, swollen and bleed easily resulting in false pocket formation. At this stage (gingivitis), the disease is still reversible and can usually be eliminated by improved oral hygiene practice. If chronic gingivitis is not treated, bacterial poisons from the plaque penetrate the deeper tissues and destroy the periodontal membrane and alveolar bone. This advanced stage of periodontal disease is known as periodontitis. In periodontitis, the junctional epithelial tissue at the base of the gingival crevice migrates down the root of the tooth resulting in deepening of the gingival pocket and true periodontal pocket formation (Levison, 1997). Serious damage to the gingivae and alveolar bone results in tooth loss (Marsh and Martin, 1992, Bragg *et al*., 1999).

The classification of periodontal diseases is still far from being resolved. Disease may be classified according to age (e.g. pre-pubertal, juvenile, adult), rate of progress (chronic, acute, rapid), and distribution of lesions (localized or generalized). Other factors which predispose to periodontal disease include hormonal changes, diabetes and HIV.

As a background to the present study, the review of the literature will focus mainly on periodontal disease associated with hormonal changes in women, with special emphasis on pregnancy and the associated risk of adverse pregnancy outcomes.

1.2. The effect of hormonal changes on women's oral health

Sex-specific relationships and the association between periodontitis and certain systemic disorders have prompted researchers to investigate the possibility of associations between periodontitis and specific women's health issues (Zakrzewska, 1996, Jeffcoat, 2000, Krejci and Bissada, 2002).

Hormonal changes during puberty, menses, pregnancy, contraceptive use and menopause all influence women's oral health and their susceptibility to periodontal disease (Steinberg, 2000; Blagojevic *et al*, 2002, Krejci and Bissada, 2002). Ovarian hormones (estrogen and progesterone) stimulate bacterial growth and promote the inflammatory process. Elevated plasma concentrations of the ovarian hormones stimulate bacterial growth and are associated with an increased incidence of gingival inflammation and exudates (Zachariasen, 1991, 1993; Soory, 2000; Tilakarantne *et al.*, 2000; America Dental Association, 2002b; Bueltmann and Stillman, 2002; Family Gentle Dental Care, 2003; Machtei *et al*., 2004). Osteoporosis (loss of bone density) has been associated with periodontal disease in postmenopausal women (Wactawski-Wende, 2001). There is some evidence that some treatments for osteoporosis, such as bisphosphonates, may reduce bone loss, including the bony structures that support the teeth (American Dental Association, 2002a).

At the present time, it would appear that more research has been done on periodontal disease associated with pregnancy than any of the other stages of the female life cycle. With mother-infant research now receiving attention because of HIV infection, other infections, such as periodontal disease, are also being examined for their impact on the well-being of the infant.

1.3. Pregnancy and periodontal disease

Pregnancy gingivitis was described at least three decades ago by (amongst others) Lundgren *et al* (1973) and Deasy and Vogel (1976) but it was not until two decades later that an association was established with adverse pregnancy outcomes, such as low birth-weight infants and preterm delivery. Sixty-five to seventy percent of all pregnant women are said to develop gingivitis around the second month of pregnancy, but the prevalence tends to decrease during the ninth month (Miyakazi *et al.,* 1991; Family Gentle Dental Care, 2003).

In a study by Rossel *et al*. (1999), 100% of pregnant women presented with some kind of gingivitis, while Tilakarantne *et al.* (2000), in a study of a rural population of Sri-Lankan women, found no statistically significant differences between the Plaque Index (PI) and loss of attachment (LA) in pregnant and non-pregnant groups, nor were differences reported with the advancement of the pregnancy. However, the Gingival Index (GI) scores of the test group increased significantly at each trimester, till the third trimester of pregnancy, when a drop in GI occurred and continued to three-months postpartum.

Sometimes, gingivae swollen by pregnancy gingivitis may react strongly to the bacteria in plaque and form large lumps. These growths, called pregnancy tumours, are not cancerous and are generally painless. Those tumours disappear after pregnancy, or if they persist, may require removal by a periodontist (American Academy of Periodontology, 2000a).

Periodontal infection of the mother may be a potential risk factor for preterm low birth-weight (PLBW) (Offenbacher *et al*., 1996; Lopez *et al*., 2002; Jeffcoat *et al*., 2001; Mitchell-Lewis *et al*., 2001). Research data suggests that 18.2% of the (PLBW) deliveries occurring each year might be attributed to periodontal disease (McGaw, 2002).

Birth is considered to be preterm if it occurs at less than 37 weeks of pregnancy, while low birth-weight is considered to be birth-weight of less than 2500g (Davenport, 1998). PLBW continues to be a significant public health issue in both developed and developing countries (McGaw, 2002). Mitchell-Lewis (2001), in his study of a cohort of young minority women in New York, found that the prevalence of PLBW was 16.5%. Mothers of PLBW infants were reported to be shorter, less educated, gained less weight during pregnancy, married to men of lower occupational class, had less healthy gingivae with more areas of bleeding and calculus (Dasanayake, 1998). After adjusting for smoking, age, race, vaginosis treatment history, marital status and previous preterm delivery, periodontal disease appeared to increase the risk of delivering a PLBW infant by more than 7 times (Offenbacher *et al*., 2001). Low birth-weight children are more likely to die during the neonatal period than infants of normal birth-weight (Shapiro *et al.,*1980) and those who survive, face a higher risk of neurodevelopmental disturbances and health problems such as asthma, respiratory infections and ear infections.

The exact mechanism by which periodontal disease may provoke PLBW has not been clearly established (Bueltmann, 2002), but it is known that the response of the mother's immune system to the infection can activate the liberation of inflammatory mediators, growth factors and cytokines which may provoke PLBW (Offenbacher *et al.,*1996). High levels of cytokines (Il-1, Il-6, and TNF-α) have been found in the amniotic fluid of patients in preterm labour (Gibbs *et al*., 1992; Offenbacher *et al.,* 1996; Dörtbudak *et al*., 2005).

1.4. The microbiology of pregnancy-associated periodontal disease

More than 500 distinct bacterial species have been reported to inhabit the periodontal pocket (Klein, 2003). Microbial species frequently associated with pregnancy-associated periodontal disease have not been discussed in much detail in the literature. Reasons for this include the fact that until recently, disease pertaining to women has been sadly neglected and of the few studies done, discrepancies occur due to differences in sampling and detection methods as well as stage of gestation (Lin *et al.*, 2003; Dörtbudak *et al*., 2005). In order to appreciate these differences, a brief outline of

microbiology detection methods deserves mention. Methods frequently used in attempting to establish the microbial aetiology of periodontal disease include differential microscopy, culture, antibody testing, molecular biology and enzyme activity. For reasons described below, many of these techniques are now being replaced by more rapid and sensitive methods of detection.

Microscopy

Darkfield and phase contrast microscopy examine plaque samples for bacterial shape and motility and are used in the quantitative assessment of bacteria such as spirochaetes, which are difficult to culture (Listegarden and Hellden, 1978; Slots *et al*., 1979; Listegarden and Levin, 1981; Singletary *et al*., 1982; Keyes and Rams, 1983; Africa and Reddy 1985a,b; Moter *et al*., 1998; Willis *et al*., 1998, Willis *et al*., 1999).

Microbial monitoring of bacterial morphotypes is a good diagnostic aid in the treatment of periodontal disease but it has its limitation in the fact that specimens have to be examined on site and immediately, for the results to have any true significance.

Fluorescence microscopy has been used to detect specific antibodies to plaque bacteria (Zambon *et al*., 1985, 1990; Drisko *et al*., 1986; Gmur, 1988). However, few specific bacteria can be detected by this technique and possibilities of false positive or false negative results can occur (Gmur, 1988).

Cultural studies

Earlier studies plated plaque samples on different growth media in anaerobic conditions. Although much information was gained regarding different species associated with periodontal disease, this technique cannot detect non-viable bacteria, nor is there a single medium which can support the growth of all the bacterial species now known to colonize the periodontal pocket (Loesche *et al*., 1984; Zambon *et al*., 1990; Africa, 1992; Brady *et al*., 1996; Chattin *et al*., 1999; Conrad *et al*., 1999; Dörtbudak *et al*., 2005).

Bacterial culture and identification is time-consuming and there are many species which have not been cultured, even though they have been successfully detected by other methods. For this reason, cultural studies are fast being replaced by other more sensitive and rapid means of detection.

Enzyme linked Immunosorbent Essay (ELISA)

The ELISA technique uses antibodies to detect antigens on target bacteria. This technique has been used to identify periodontopathic microflora. But in some cases of spirochaetes, the relationship between elevated antibody titres and disease activity is still unclear because infected subjects could have elevated titres due to past infection (Genco *et al.*, 1986; Kornman, 1987).

DNA Probes

DNA probes have a strong interaction with a specific target only, and can be detected by colorimetric or chemiluminescence techniques (Keller and Manak, 1991). These methods are rapid and can detect nonviable species (Loesche *et al*., 1992; Tenenbaum *et al*, 1997; Takamatsu, 1997).

Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) is a technique used to amplify the number of copies of a specific region of DNA, in order to increase the yield of DNA in a sample**.** It can be used with huge accuracy in disease identification and forensic investigations. Two oligonucleotide primers are used that hybridise to opposite strands and flank both ends of a given region of DNA (Erlich, 1989, White, 1993). Many studies have employed PCR to detect individual species in subgingival plaque (Watanabe and Frommel, 1993; Guillot and Mouton, 1996; Tran and Rudney, 1996; Watanabe and Frommel, 1997; Conrads *et al*., 1999; Takamatsu, 1997; Willis *et al*., 1999; Tran and Rudney, 1999; Doungudomdacha *et al*., 2000; Kuhnert *et al*., 2002; Edwards *et al*., 2003; Klein *et al*., 2003). Compared with culture, PCR is rapid, sensitive (can detect < 10^3 bacteria) and

more accurate (Siqueira, 2001). PCR is also able to detect bacterial species which are difficult or impossible to culture.

Enzyme tests

Porphyromonas gingivalis, Tannerella forsythensis, previously called *Bacteroides forsythus* (Sakamoto, 2002) *and Treponema denticola* are among the few subgingival microflora associated with periodontal disease which possess a trypsin-like enzyme capable of hydrolyzing the synthetic peptide N-benzoyl-DL-arginine-B-naphthylamide, commonly referred to as BANA (Laughon *et al*., 1982; Loesche *et al*., 1990). The commercially available BANA test (Oral B, Perioscan^{TM}) is a modification of the BANA hydrolysis test adapted from the trypsin-like enzyme of the API-ZYM Kit (Laughon *et al.*, 1982; Loesche *et al.*, 1990). BANA is a rapid and effective diagnostic aid and has shown to correlate well with the clinical indices used to diagnose periodontal disease (Loesche *et al*., 1990;1997a,b; Grisi *et al*., 1999, 2001). BANA is able to detect between 10^3-10^5 targets/sample (Loesche *et al.*, 1992; Tran *et al*., 1999) which is within the same range as immunoassays and nucleic acid probes.

SK-013 (carbobenzoxy-glycyl-arginine-3,5-dibromo-4-hydroxy-arginine) has also been used for the detection of peptidase activity of *T.denticola, P. gingivalis and T. forsythensis* in plaque samples (Ishihara *et al.,* 1991). However, BANA appears to be more frequently employed.

Results obtained using the above microbiological assays have suggested a synergy between plaque bacteria co-habiting the same site (Onagawa *et al*., 1994; Yao *et al*., 1996; Socransky *et al*., 1998). Earlier studies (Kornman and Loesche, 1980) found that during pregnancy, the ratio of bacterial anaerobes to aerobes and the proportions of *Bacteroides melaninogenicus, Prevotella intermedia (Bacteroides intermedius)* and *Porphyromonas gingivalis (Bacteroides gingivalis)* increased. Pregnant women were found to have a level of *Bacteroides* species 55 times higher than that of non-pregnant women (Jensen *et al.*, 1981).

Pregnancy, because it is an immune-hyporesponsive state, appears to increase a women's susceptibility to infection. This, in turn, puts the foetus at risk for congenital or neonatal infections, resulting in both maternal and foetal morbidity and mortality. Although physical and immunological barriers protect the foetus from most infections while in the uterus, some microbes are able to cause transplacental or congenital infections via haematogenous spread from the mother, while others colonise the infant at delivery, during its passage through the birth canal. Exogenous pathogens such as *Treponema pallidum*, Rubella, Varicella, Herpes Simplex Virus, *Listeria monocytogenes* etc.**,** have received more attention for the role they play in congenital and neonatal infections than endogenous organisms such as Group B streptococci (GBS) and some of the suspected periodontopathogens such as *Treponema denticola*, *Porphyromonas gingivalis* and *Tannerella forsythensis*. Diagnostic tests such as BANA-hydrolysis, have confirmed their role as indicators of infection (Loesche *et al.*, 1990) and their strong association with severe periodontal disease, led to the designation of this group of bacteria as the **red complex** (Socransky *et al*., 1998)**.**

Using statistical analysis, Socransky *et al.,* (1998) clustered frequently occurring bacterial species into complexes which were colour-coded in order to facilitate discussion (Table 1). The **red complex** was found to be most closely associated with pocket depth and bleeding on probing, while the **orange complex** related to pocket depth but less frequently with the other clinical parameters used in the diagnosis of periodontal disease.

Table 1: Bacterial clusters described by Socransky *et al* (1998).

Complex	Bacterial cluster
Red	*Treponema denticola*, *Porphyromonas gingivalis*, *Tannerella forsythensis*
Orange	*Fusobacterium nucleatum, Prevotella intermedia, Prevotella nigrescens*, *Peptostreptococcus micros* (Associated species included) *Eubacterium nodatum, Campylobacter rectus, Campylobacter showae,* *Streptococcus constellatus, Campylobacter gracilis*)
Yellow	*Streptococcus sanguis, Streptococcus oralis, Streptococcus mitis, Streptococcus gordonii, Streptococcus intermedius*
Green	Carpnocytophaga, *Camplobacter concisus*, *Eikenella corrodens*, *Actinobacillus actinomycemcomitans* (serotype a)
Purple	*Veillonella parvula*, *Actinomyces odontolyticus*, Actinobacillus actinomycemcomitans (serotype b), *Selenomonus noxia*, *Actinomyces naesludii*

1.5. Association of the "red complex " with adverse pregnancy outcomes

Serum Antibody levels to suspected periodontopathogens are useful markers for periodontal infection (Ebersol, 1982). Blood samples from foetal cords tested for the presence of IgM antibody against 13 periodontal pathogens, showed that among the PLBW samples, 33.3% tested positive while only 17.9% of the normal birth-weight samples tested positive (Offenbacher *et al.* 1999). Of the 13 periodontal pathogens used in the research, IgM antibodies against *C. rectus, P.gingivalis and F. nucleatum* were most often encountered. Dasanayake *et al.* (2001**,** 2003) found that women with higher levels of *P. gingivalis*-specific IgG had higher odds of giving birth to PLBW infants and this association remained significant after controlling for smoking, age, race and IgG

levels against other selected periodontal pathogens**.** Madianos *et al*.,(2001) reported that mothers with lack of maternal IgM antibody to the "Red Complex**"** were found to have a higher rate of preterm deliveries and they proposed that circulating antibodies had a protective function in pregnant women.

Offenbacher *et al.* (1996) reported high levels of *B. forsythus (T. forsythensis), P.gingivalis, A. actinomycetemcomitans and T. denticola* in mothers who delivered pre-term, low-birth-weight infants. A study of women at risk for pregnancy complication revealed that the "Orange Complex" and the "Red Complex**"** were found in 18% of full-term and 100% of preterm cases. High counts of CFU of periodontopathic bacteria were found in 5/6 cases in women who delivered preterm and only 4/30 in women who delivered full-term (Dörtbudak *et al*., 2005).

The BANA test is indicated for use in patients with known risk factors for periodontal disease such as smokers and those at risk for coronary artery disease (De Stefano *et al*., 1993). There is no documented proof that BANA has been used as a screening test for mothers at risk for adverse pregnancy outcomes**.** Since the **red complex** has been implicated in adverse pregnancy outcomes**,** it would be interesting to establish whether the BANA test could be used to identify mothers at risk for preterm delivery or low birth-weight infants. Early detection of periodontopathic bacteria could lead to adequate therapy, thus eliminating the risk of inflammation and infection dissemination. Most studies addressing stillbirth, preterm birth and low birth-weight babies have been performed in USA (Offenbacher 1996; Jeffcoat *et al*., 2001, 2003; Davenport *et al***.,** 2002; Lopez *et al*., 2002) and to our knowledge, nothing has been reported in the literature regarding endogenous oral pathogens and adverse pregnancy outcomes in South Africa. This study will therefore be the first in South Africa to screen patients at risk for adverse pregnancy outcomes using the BANA technique and PCR.

1.6. Summary and objectives

It is only recently, that research has focused on issues relating to women's health and the possible threat to their infants. Although isolated studies have established some association of changes in microbial flora in different stages of the female life-cycle, there are many unanswered questions relating to the effect oral disease can have on the unborn child. For this reason, a closer look at the microbiology of pregnancy periodontal disease is needed, and rapid, sensitive methods are needed to detect periodontal disease in pregnant mothers thus identifying those at risk of delivering preterm or low birth-weight infants. To our knowledge no studies have been reported in the literature testing the efficacy of BANA as a screening test for adverse pregnancy outcomes, nor has anything been published associating the **red complex** with pregnancy, using PCR.

The objectives of this study are therefore to examine the prevalence of *Treponema denticola*, *Tannerella forsythensis*, and *Porphyromonas gingivalis* in subginginval plaque samples from pregnant women in order to:

- observe the oral health status of pregnant women attending the MOU in order to establish the prevalence of the red complex using BANA and PCR,
- evaluate the use of BANA as a screening test for mothers at risk of pre-term delivery or low birth weight.

CHAPTER 2: MATERIALS AND METHODS

2.1. Sample selection

The study sample consisted of pregnant women attending the early morning clinic at a local Maternity Obstetrics Unit (MOU) in Mitchells Plain, Western Cape. Their ages ranged between 17 and 45 years and their selection was random. No instruction for oral hygiene had preceded the measurements or collection of samples.

2.2. Ethical consideration

For the sake of convenience, the appendix numbers will correspond with the chapter number in this text e.g. 2A, 2B etc. Informed consent was obtained from the participants in verbal and written form as defined by the Medical Research council, 2001 (see appendix 2A). They were informed of the purpose of the study, the benefits and potential risks and their right to refuse participation (see appendix 2B). After written informed consent was obtained, study participants completed a questionnaire containing items designed to obtain information regarding medical history, factors predisposing for periodontal disease, and oral hygiene habits (see appendix 2C).

2.3. Clinical assessment

The oral examination was done by Dr Soraya Harnekar (Faculty of Dentistry, UWC).
The clinical assessment included patient history, recording of clinical indices and oral lesions (if present).
Decayed, missing, and filled (DMF) teeth were recorded (Ndiaye *et al*., 1997). Interproximal sites of the six Ramfjord teeth were used in the clinical examination (Ramfjord, 1959; Gettinger *et al*., 1982). The Ramfjord teeth are:

- Maxillary right first molar (16)
- Maxillary left central incisor (21)
- Maxillary left first bicuspid (24)
- Mandibular left first molar (36)

- Mandibular right central incisor (41)
- Mandibular right first bicuspid (44)

Measurements were taken at the mesial locations of the selected teeth.

Missing teeth were substituted as follows:

16: by the mesial of 15, if not by the distal of 17

21: by the distal of 22

24: by the distal of 25

36: by the mesial of 35, if not by the distal of 37

41: by the distal of 42

44: by the distal of 45.

The amount of plaque present (PI) was assessed using the criteria of Silness and Löe (1964) and scored from 0-3 (Table 2).

Table 2- <u>The Plaque Index System</u> (Silness and Löe, 1964):

Criteria	**Scores**
No plaque	0
A film of plaque adhering to the free gingival margin and adjacent area of the tooth. The plaque may be seen only by using the probe on the tooth surface.	1
Moderate accumulation of soft deposits within the gingival pocket, or on the tooth and gingival margin which can be seen with the naked eye.	2
Abundance of soft matter within the gingival pocket and or on the tooth and gingival margin.	3

Gingival inflammation (GI) was scored from 0-3 according to the method of Löe and Silness (1963). This index is based upon the clinical characteristics of the different stages of the gingival inflammation (Table 3).

Probing depth (PD) was measured from the gingival margin to the base of the pocket using a periodontal probe and loss of attachment (LA) was measured from the cemento-enamel junction to the base of the pocket.

Table 3- Criteria and scores for the Gingival Index System (Löe and Silness, 1963)

Criteria	Scores
No inflammation	0
Mild inflammation- slight change in color and little change in texture.	1
Moderate inflammation- moderate glazing, redness, oedema, and hypertrophy. Bleeding on probing.	2
Severe inflammation, redness and hypertrophy. Spontaneous bleeding. Ulceration.	3

2.4. Subgingival plaque sampling

After removal of supragingival plaque, subgingival plaque was collected by inserting a sterile periodontal probe into the base of the pocket. The samples were dispersed in vials containing 500 µl of sterile saline (0.75%) with 5 glass beads (to assist in dispersing sample) and transported directly to the laboratory.

2.5. Assay for BANA-Hydrolyse activity

The BANA test was done according to the manufacture's instructions (Perioscan, Oral B). Briefly the principle of the test is as follows. The BANA hydrolysis test is a plastic card with two separate reagent matrices (strips). The lower strip is impregnated with BANA reagent and the upper strip contains a chromogenic diazo reagent, Fast Black K. B-naphthylamide, one of the hydrolytic products of the BANA reaction reacts with the Fast Black K, producing a permanent blue colour. Site information was recorded on the BANA Test card in the marked space.

The samples were dispersed for 10 seconds with a vortex mixer (Heidolph vortex, Rose Scientific Ltd). Aliquots of 150 µl were centrifuged at 3000Xg for 10 min in an eppendorf tube (Eppendorf Centrifuge 5415 C, Daves Electronics Plus) and 7 µl of the pellet (Loesche *et al*., 1992) was applied to the lower reagent strip of the BANA Test card. The remaining sample was frozen till further use. The upper strip of the Perioscan

card containing the fast black dye was activated by moistening with distilled water, the BANA card folded at the perforation (so that the white lower and buff upper matrice came in contact with each other) and placed in the BANA incubator for 15 min at 55^0 C. The BANA test results were read on the upper buff matrix and the results recorded on the patient's chart as negative (no blue colour), or positive (blue colour). A positive result suggested that the plaque contained at least 10^4 to 10^5 BANA-positive organisms (Loesche *et al*., 1990). Positive controls of *T. denticola* (ATCC 33520) were used to ensure that the test cards were working.

2.6. Polymerase Chain Reaction

2.6.1. Primer design and selection

Species-specific primers (Inqaba Biotech Industries (Pty) Ltd) were used to detect the presence of the "red complex". The expected product lengths were 641 bp for *T. forsythensis*, 404 bp for *P. gingivalis* and 316 bp for *T. denticola.* A pair of ubiquitous primers product length (602 bp) which matched most bacterial 16S rRNA genes at the same position was used as a positive control for the PCR reaction (Rôças *et al*., 2001). Nucleotide sequences of selected and modified 16S rDNA primer pairs. (Rôças *et al*., 2001) are listed in Table 4.

Table 4: **PCR primer sequences used for detection of red complex**

Target	**PCR primer pairs (5'-3')**
Tannerella forsythensis: - Forward	GCG TAT GTA ACC TGC CCG CA
- reverse	TGC TTC AGT GTC AGT TAT ACC T
Porphyromonas gingivalis: - Forward	AGG CAG CTT GCC ATA CTG CG
-Reverse	ACT GTT AGC AAC TAC CGA TGT
Treponema denticola: - Forward	TAA TAC CGA ATG TGC TCA TTT ACA T
- Reverse	TCA AAG AAG CAT TCC CTC TTC TTC TTA
Ubiquitous primers: - Forward	GAT TAG ATA CCC TGG TAG TCC AC
- Reverse	CCC GGG AAC GTA TTC ACC G

2.6.3. Preparation of reference DNA

Initially, two methods were used in order to establish which produced a greater yield of DNA.

Method A

Bacterial cultures were centrifuged at 4000 rpm for 10 minutes at 4^0 C, washed in Tris Hydroxymethyl Methylamine (TES see appendix 2E**)** buffer, resuspended in 20 ml TES and stored at -70^o C.

Cell were lysed by the addition of 1 mg/ml lysozyme and incubated for 10 minutes at 37^o C or until changes in colour or viscosity were seen. Lipid molecules were removed by the addition of sodium dodecylsulphate detergent (SDS see appendix 2E) to a final concentration of 1 %, followed by another incubation at 37^o C for 10 minutes.

Proteinase K (1 mg/ml, dissolved in 0.5 M Tris-HCl pH 7.2) was added and DNA incubated at 37^o C for 30 minutes. Proteinase K was added to facilitate the breakdown of protein molecules and facilitate phenol extraction. Protein was precipitated and extracted with phenol/chloroform as follows: a volume of 500 µl of the aqueous solution of DNA was added to 250 µl phenol solution and 250 µl chloroform, gently mixed by inversion for 1 minute, then spun at 4000 rpm for 10 minutes. Because phenol and water are immiscible, the protein was extracted into the phenol layer (bottom layer), leaving nucleic acids in the aqueous top layer. The aqueous top layer containing nucleic acids was removed. The extraction procedure was repeated twice.

DNA was precipitated by the addition of 50 µl of 5M NaCl and 1 ml of 100 % ethanol. After chilling on ice for 3-5 minutes, the tube was gently mixed by inversion and spun for 10 minutes at 4^o C. If the high molecular weight nucleic acids were not precipitated as a white fibrous material, but rather as a colourless, gelatinous material, it indicated that protein was still bound to the nucleic acid. The nucleic acid was then treated with

ribonuclease (RNase) at 65° C for 10 minutes (10 mg in 1 ml TE buffer), followed by another round of proteinase K treatment, phenol/chloroform extraction and ethanol precipitation. The pellet was washed in 70% ethanol without centrifugation (just poured off without mixing) and resuspended in 500 µl sterile distilled water. Fifty µl 5M ammonium acetate (see appendix 2E) was added, followed by 1 ml cold ethanol. The solution was mixed by inversion and left to precipitate at -20° C for 5 minutes. The DNA was carefully spooled with a glass rod and washed by dipping in 500 µl 70% ethanol, and then in 100 µl TE. The DNA was left overnight at 37° C, if not dissolved immediately.

If the DNA preparation appeared very dilute, it was concentrated as follows:
300 µl DNA was mixed with 30 µl 5M NaCl and 750 µl cold ethanol, incubated at -20° C for at least an hour, then spun for 10 minutes. The supernatant was removed and the pellet washed with 70% ethanol, dried under vacuum and resuspended in 100 µl TE. This phase was re-extracted with 600 µl TE. If method A failed to yield an adequate amount of DNA, method B was used.

Method B

Cells were harvested at 7000 rpm at 4° C for 15 minutes, resuspended in 1 ml TE. After spinning for one minute speed, the pellet was resuspended in 300 µl buffer consisting of 100 µg/ml RNase A in 50 mM Tris-Cl, 10 mM EDTA (pH 8.0). One mg of lysozyme in 50 µl distilled water was added, followed by incubation at 37° C for 20 minutes. Seventeen µl of 20% SDS to a final concentration of 1 % was added and the solution incubated at 37° C for 10 minutes. The final volume was made up to 600 µl with TE. The solution was mixed by inversion and extracted with TE buffered phenol, leaving behind the sheet of bacteria debris at the interphase. This was followed by precipitation of the combined aqueous phase with a tenth of the volume (60 µl) of 5M ammonium acetate and 2 volumes (1.2 ml) of cold ethanol (-20° C). DNA was spooled with a glass rod, washed in 70% ethanol, resuspended in 500 µl TE and allowed to dissolve slowly.

Table 5- Preparation of the PCR cocktail

	Initial conc.	Final conc.	Volume/µl for 1 tube
F primer	40 µM	40 µM	1
R primer	40 µM	40 µM	1
BufferX10	X10	X1	5
$MgCl_2$	25 mM	1 mM	4
dNTP	20 mM	0.2 mM	0.5
DNA	-	-	5
Taq polymerase	250 U	1.25 U	0.25
dH_2O	-	-	33.25
Total	**-**	**-**	**50**

The *Taq*, $MgCl_2$ and 10 x buffer were supplied by (JMR-801, Southern Cross Biotechnology) and the dNTP was supplied by (AB-0196, Southern Cross Biotechnology). Dilution of primer were done using 1 X TE (see appendix 2E).

2.6.3. Preparation of samples for PCR

Samples were prepared for PCR accoding to the method of (Conrads *et al*., 1999). Deep-frozen 250-µl suspensions of samples were thawed by incubation for 10 min at 37^0 C. Samples were vortexed for 30 seconds and centrifuged for 2 minutes at 2500X g. The supernatant was removed and the pellet resuspended in 100 µl of distilled water. Another step of vortexing and centrifugation was done and the pellet resuspended in 500 µl of distilled water. The suspension was heated for 10 min at 94^0 C (Omeg HB/01 digital heating block, Omeg Scientific) and the vials immediately chilled on ice for 5 min. After centrifugation for 10 seconds at 9000X g, 5 µl aliquots of the supernatant was used in the PCR assay. This was added to 45 µl of the PCR cocktail mix (see Table 5). The blank sample (negative control) containd 5 µl of dH_2O in place of the sample, while the positive control consisted of 49 µl from the cocktail mix and 1 µl of the reference genomic DNA. Samples were mixed by vortex briefly and layered with 20µl of mineral

oil. Amplification of samples was achieved using thermal cycle (HYBAID, Omnigene temperature Cycling System) under the conditions listed in Table 6 (Rocas *et al*., 2001).

Table 6: Conditions of PCR amplification

	Principles	*P. gingivalis*	*T.denticola, T.forsythensis* and ubiquitous
Initial denaturation (1 cycle)	DNA denatures to form a single stranded DNA	95^0 C for 2 minutes	95^0 C for 2 minutes
Denaturation (1 cycle)		94^0 C for 30 seconds	95^0 C for 30 seconds
Annealing (36 cycles)	Primers bind to the template DNA	60^0 C for 1 minute	60^0 C for 1 minute
Extention (1 cycle)	New DNA strands built	72^0 C for 2 minutes	72^0 C for 1 minute
Final extention (1 cycle)		72^0 C for 10 minutes	72^0 C for 2 minutes

2.6.4. Detection of PCR products by agarose Gel Electrophoresis with Ethidium Bromide staining

The size and purity of the PCR products were determined by electrophoresis in 1.5% agarose gel (Midi ABagarose AG-0300/b) using Tris-borate-EDTA buffer (1XTBE, see appendix 5). The agarose was melted in a microwave until dissolution of all particules and was cooled down before adding 2 µl of ethidium bromide (0.5 µg/ml, see appendix 5). After solidification of the agarose gel, it was put in the electrophoresis tank (MAX Submarine agarose Gel Unit, Model HE 99) and covered with 1X TBE. Five µl of loading buffer (see appendix 5) was added to 50 µl of each PCR sample and loaded into the gel. The first well of the agarose gel, contained the 100bp marker (Supperladder-Low 100 bp size marker, SLL-100S), well 2 the positive control, well 3 the negative control (water) and the remaining wells contained DNA test samples. The electrophoresis was

performed at 90 V for one hour. The DNA was visualized under ultraviolet illumination (UV TRANSILLUMINATOR UVP, INC). The specificity of the PCR was evaluated by testing representatives of related and unrelated bacterial species. These included members of the **red complex** as well as, *Fusobacterium nucleatum*, *Prevotella intermedia, Escherichia coli, Bacteroides fragilis, Bacteroides vulgatus*, *Porphyromonas asaccharolyticus*, *Treponema socranskii*.

2.7. Data Analysis

Data was analyzed using Epi Info, a microcomputer statistics program produced by the Center for Disease Control, Atlanta, Georgia and the World Health Organization, Geneva. All questionnaires, oral examination and laboratory data were entered into the program and statistical analysis were performed by Theo Data Statistical Consultation Services (Belville, Cape Town) using Excel and the Number Cruncher Statistical System (NCSS). Spearman Rank Correlation and Kruskal-Wallis One-Way ANOVA were used to determine significant correlations.

The accuracy of the test was expressed by using the following formula:
Sensitivity: TP/(TP+ FN), Specificity: TN/(FP + TN), positive predictive value (PPV): TP/(TP + FP), negative predictive value (NPV): TN/(FN + TN), Positive odds ratios: Sensitivity/1 – specificity while negative odds ratios were obtained by the following formula: FN/TP + FN divided by TN/FP +TN. The percentage accuracy for each method was calculated using the formula: TP + TN/sample number X 100.
TP: True positive, TN: true negative, FN: false negative, FP: false positive.

CHAPTER 3: RESULTS

3.1 Results

3.1.1. Health Risk Assessment Questionnaire

Prior to the oral examination, mothers were asked to complete a questionnaire in order to identify factors which might pose a health risk to them and their infants. The responses to the questionnaire are summarized in Table 7. All the participants had received some level of education. Most had completed high school (89.4%), followed by primary school (6.1%) and university education (4.5%). Seventy-seven percent lived in a house, 16.7% in shacks and 6.1% lived in flats. Sixty-seven percent lived 5 or less than 5 people in a home, while 32.7% of them lived more than 5 people in a home. Thirty-seven percent were in their first pregnancy, while 63.1% had already two or more pregnancies.

Of the patients examined, 78.8% were HIV-seronegative, 7.6% were HIV-seropositive while the remaining 13.6% were not willing to be tested for HIV. Forty-six percent visited a gynecologist when necessary, while 37.9% never had routine gynecological check-ups prior to pregnancy. Annual check-ups were claimed by 10.3% and 5.2% claimed that they had monthly check-ups. Eighty-five point five % and 86.2% reported not having a urinary tract infection or a sexually transmitted disease respectively.

Thirty-two % reported never visiting a dentist while 40.7% visited a dentist only when necessary. Annual visitations were reported by 20.3% and bi-annual visitations by 6.8 %. The majority (63%) reported brushing their teeth twice a day and 25.9% brushed once a day with only 11.1% brushing after every meal. Only 20.4% admitted to having bad breath with 79.6% responding negatively.

Half of the participants reported having easy access to medical and dental care but only 6.2% had medical insurance. One third (32.3%) admitted to smoking and 27.0% admitted to alcohol consumption on special occasions only.

Forty-eight of the sample population reported to have fruit and vegetables as main diet, 21.4 % bread, 12.5 % meat and 10.8 % bread, fruit and vegetables.
No diabetics were included in our sample population because they attended the clinic on Fridays and we collected samples on the other days of the week.

3.1.2. Oral hygiene status

Three hundred and seventy-two samples were collected from 66 pregnant women attending the Mitchells Plain's MOU. We examined the overall hygiene status of each patient. Figures 1-5 demonstrate the oral status of 5 of the patients examined. Only 11 (16.9 %) of the subject group had no decayed teeth, while greater than 80 % had one or more missing teeth and only 15 % had fillings (Table 8). One third (33.84 %) of the subject group had more than seven teeth missing. Thirty-five % had between 3-6 missing teeth, 21.53 % had between 1-2 missing teeth and only 9.23 % had no missing teeth. Almost 68 % had a DMF-T greater than 7 and 20 % had a DMF-T between 3 and 6.

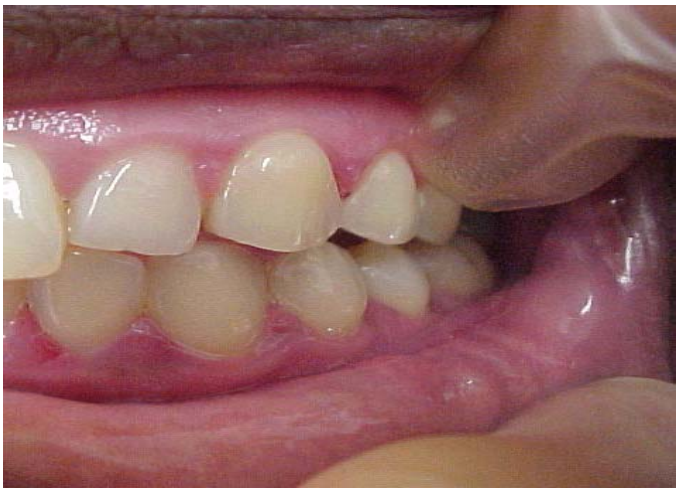

Figure 1: Healthy gingiva around the central incisors; gingival papillary hyperplasia present in the posterior and lower anterior segments.

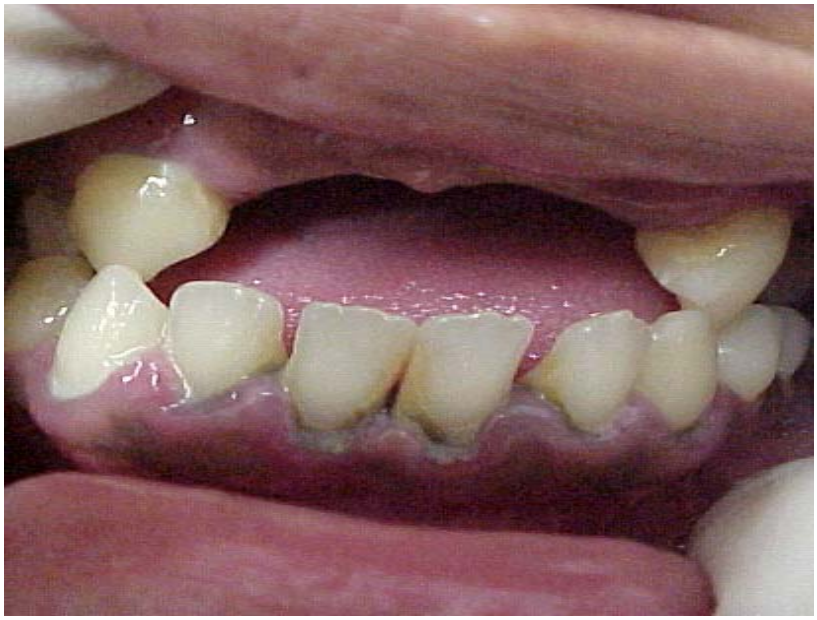

Figure 2: Patient with upper central incisor teeth extracted; generalized moderate-severe gingival inflammation; subgingival calculus deposits; loss of gingival contour in the lower anterior segment.

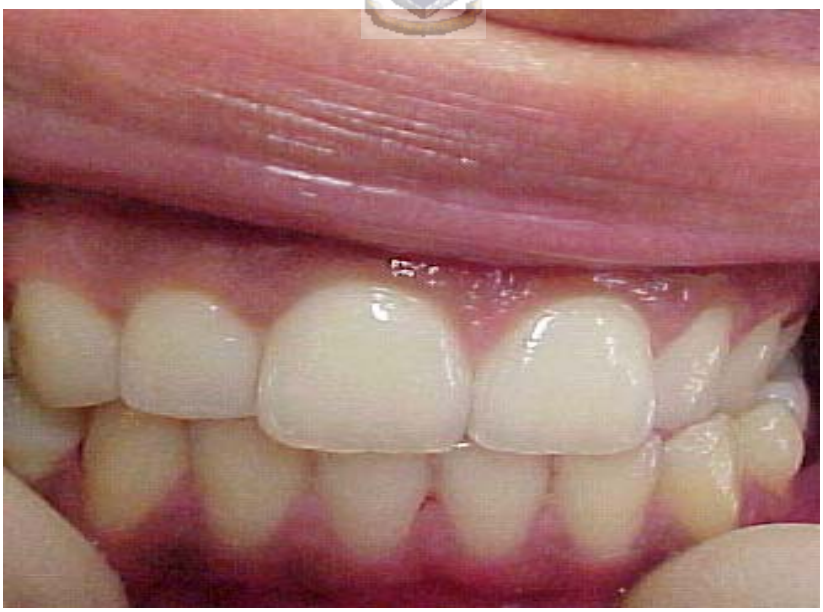

Figure 3: Healthy mouth and gingival tissues with a visible cross bite on the left side.

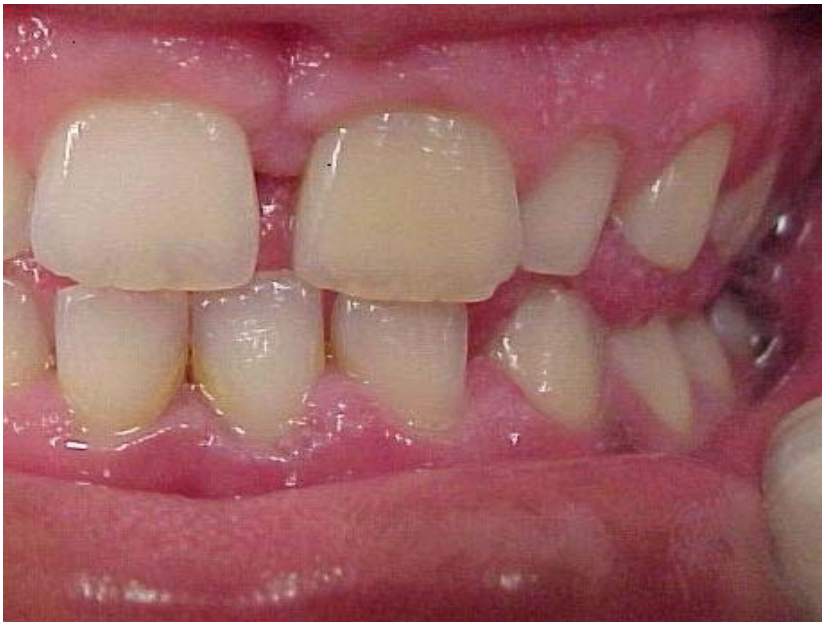

Figure 4: Patient with generalized moderately fibrotic appearance of the gingiva.

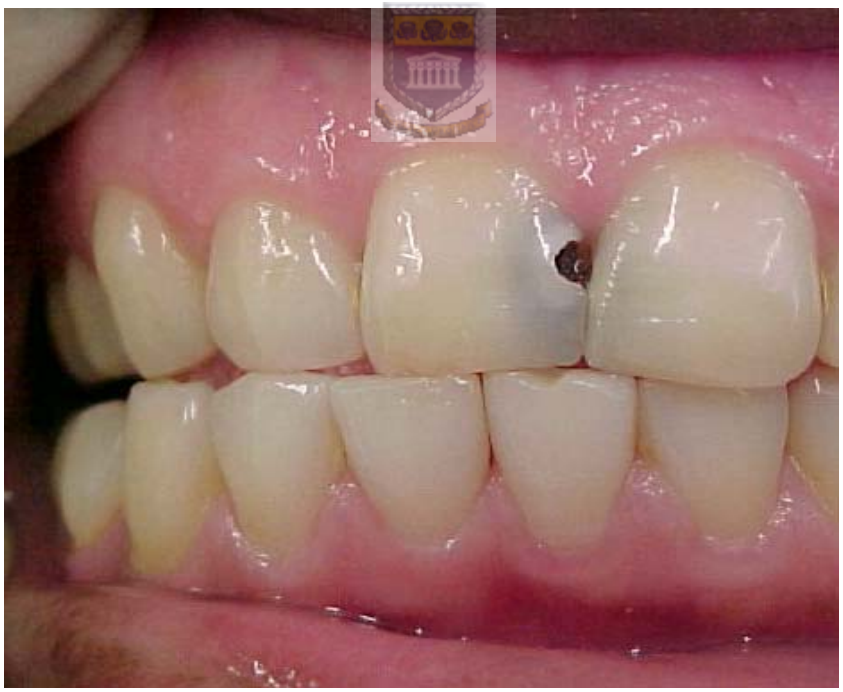

Figure 5: Patient with gingival tissue mildly fibrous in appearance and caries on the 11 mesial.

Table 7: Health Risk Assessment Questionnaire

Characteristics	Frequency	Percent
1. LEVEL OF EDUCATION		
- **University**	3	4.5 %
- **High school**	59	89.4 %
- **Primary school**	4	6.1 %
- **No formal education**	0	0 %
2. LIVING CONDITIONS:		
NUMBER OF PEOPLE IN A HOME		
- **≤ 5**	35	67.3 %
- **> 5**	17	32.7 %
HABITATION		
- **Flat**	4	6.1 %
- **House**	51	77.3 %
- **Shack**	11	16.7 %
3. HISTORY OF PREVIOUS PREGNANCIES		
FIRST PREGNANCY		
- **Yes**	24	36.9 %
- **No**	41	63.1 %
INDUCED ABORTION		
- **Yes**	3	9.1 %
- **No**	30	90.9 %
4. HIV STATUS		
- **HIV-Seronegative**	52	78.8 %
- **HIV-Seropositve**	5	7.6 %
- **Not ready**	9	13.6 %
5. GYNECOLOGICAL HISTORY:		
GYNECOLOGICAL CHECK-UPS		
- **Never**	22	37.9 %
- **Monthly**	3	5.2 %
- **Once a year**	6	10.3 %
- **Only when necessary**	27	46.6 %
URINARY TRACT INFECTION		
- **Yes**	9	14.5 %
- **No**	53	85.5 %
SEXUALLY TRANSMITTED DISEASE		
- **Yes**	9	13.8 %
- **No**	56	86.2 %

Table 7 (continued)

Characteristics	Frequency	Percent
6. DENTAL HISTORY:		
VISIT TO A DENTIST		
- **Never**	19	32.2 %
- **Once a year**	12	20.3 %
- **Twice a year**	4	6.8 %
- **Whenever**	24	40.7 %
FREQUENCY OF BRUSHING		
- **Once a day**	14	25.9 %
- **Twice a day**	34	63.0 %
- **After every meal**	6	11.1 %
BAD BREATH		
- **yes**	10	20.4 %
- **No**	39	79.6 %
7. MEDICAL CARE		
Medical or dental care		
- **Yes**	29	50 %
- **No**	29	50 %
Medical insurance		
- **Yes**	4	6.2 %
- **No**	61	93.8 %
8. CIGARETTE AND ALCOHOL USE		
Current smokers		
- **Yes**	21	32.3 %
- **No**	44	67.7 %
Alcohol use		
- **Never**	45	71.4 %
- **Special occasion**	17	27.0 %
- **Weekly**	1	1.6 %
MAIN DIET		
- **Bread**	12	21.4 %
- **Meat**	7	12.5 %
- **Bread and Meat**	1	1.8 %
- **Fruit and veg**	27	48.2 %
- **Bread, Fruit and veg**	6	10.8 %
- **Meat, Fruit and veg**	1	1.8 %
- **Everything**	2	3.6 %

Table 8: Decayed, Missing and Filled teeth

Characteristics	Frequency	Percent
DECAYED TEETH		
- **None**	11	16.92 %
- **1-2**	15	23.07 %
- **3-6**	28	43.07 %
- **› 7**	11	16.92 %
MISSING TEETH		
- **None**	6	9.23 %
- **1-2**	14	21.53 %
- **3-6**	23	35.38 %
- **› 7**	22	33.84 %
FILLED TEETH		
- **None**	55	84.61%
- **1-2**	4	6.15 %
- **3-6**	6	9.23 %
- **› 7**	0	0 %
DMF-T		
- **None**	4	6.15 %
- **1-2**	4	6.15 %
- **3-6**	13	20 %
- **› 7**	44	67.69%

3.1.3. Distribution of Age and Stage of Pregnancy

The age of the study population ranged from 17 to 45 years with a mean of 26.7 years (Table 9). Half (50%) were aged between 17-25 years. Only one patient was in the first trimester of pregnancy, 30.3% were in the second trimester and 68.8% in the third trimester of pregnancy.

Table 9: Distribution of Age and Stage of Pregnacy

Characteristics	Frequency	Percent
AGE		
Main age : 26.7 (±6.49)		
- **17-25 Years**	33	50 %
- **26-30 Years**	13	19.6 %
- **31-35 Years**	13	19.6 %
- **36-40 Years**	5	7.5 %
- **≥ 41 Years**	2	3.0 %
STAGE OF PREGNANCY		
- **1st Trimester**	1	1.5 %
- **2nd Trimester**	20	30.3 %
- **3rd Trimester**	45	68.8 %

3.1.4. Periodontal indices

Of the 372 sites examined, 68.5% had a PI score equal to 0 with a mean PI of 0.4 (Table 10). The mean GI score was 1.04 (±0.54) with 78.3% measuring GI ≥1. The mean pocket depth was 3.2 mm (±0.75). Sixty-six percent of the 372 sites measured, had a PD score between 1 and 3mm and 33.1% had a PD between 4 and 6mm. Because the majority of sites showed no loss of attachment, this made the data unsuitable for statistical analyses. The PI, GI and PD for each patient were obtained by adding the PI, GI and PD respectively for all six Ramfjord teeth and dividing by six. Appendix 3A summarizes the details of all the clinical indices according to the different Ramfjord teeth.

Table 10: Means and Standard deviation (SD) of Clinical Indices

	Number of sites	Percent
Plaque Index		
- **0**	255	68.5 %
- **1**	95	25.5 %
- **2**	22	5.9 %
- **Mean (SD)**	0.4 (±**0.47)**	
Gingival Index		
- **0**	80	21.5 %
- **1**	204	54.9 %
- **2**	85	22.9 %
- **3**	2	0.5 %
- **Mean (SD)**	1.04 (±**0.54)**	
Pocket Deph		
- **1-3 mm**	244	66.3 %
- **4-6mm**	122	33.1 %
- **> 6 mm**	2	0.5 %
- **Mean (SD)**	**3.2 (±0.75)**	

3.1.5. The relationship between periodontal indices and pregnancy

Only 1 patient enrolled in the first trimester of pregnancy, her clinical indices measured higher than the means for those in the second and third trimesters (Table 12). This study showed no significant difference between the mean PI of women in the second (0.40 ± 042) trimester and those enrolled in the third trimester (0.38 ± 0.46). However, the mean GI and PD scores increased from the second to the third trimester of pregnancy.

Table 11: Spearman Rank Correlation

		A	B	C	D	E	F	G	H	I
A	r	1	-0.188	0.277	-0.228	0.031	-0.019	0.274	0.156	-0.052
	P	0	0.181	0.033	0.065	0.804	0.88	0.03	0.21	0.678
	n	66	52	59	66	66	66	63	66	66
B	r	-0.188	1	-0.345	-0.221	-0.146	0.048	0.034	0.05	-0.037
	P	0.181	0	0.017	0.116	0.302	0.736	0.816	0.724	0.794
	n	52	52	47	52	52	52	49	52	52
C	r	0.277	-0.345	1	-0.108	-0.03	-0.149	-0.017	-0.006	-0.087
	P	0.033	0.017	0	0.416	0.82	0.259	0.903	0.965	0.514
	n	59	47	59	59	59	59	56	59	59
D	r	-0.228	-0.221	-0.108	1	0.491	0.412	0.214	0.132	0.336
	P	0.065	0.116	0.416	0	0	0.001	0.092	0.29	0.006
	n	66	52	59	66	66	66	63	66	66
E	r	0.031	-0.146	-0.03	0.491	1	0.506	0.22	0.268	0.396
	P	0.804	0.302	0.82	0	0	0	0.084	0.03	0.001
	n	66	52	59	66	66	66	63	66	66
F	r	-0.019	0.048	-0.149	0.412	0.506	1	0.227	0.263	0.227
	P	0.88	0.736	0.259	0.001	0	0	0.073	0.033	0.067
	n	66	52	59	66	66	66	63	66	66
G	r	0.274	0.034	-0.017	0.214	0.22	0.227	1	0.168	0.111
	P	0.03	0.816	0.903	0.092	0.084	0.073	0	0.187	0.385
	n	63	49	56	63	63	63	63	63	63
H	r	0.156	0.05	-0.006	0.132	0.268	0.263	0.168	1	0.518
	P	0.21	0.724	0.965	0.29	0.03	0.033	0.187	0	0
	n	66	52	59	66	66	66	63	66	66
I	r	-0.052	-0.037	-0.087	0.336	0.396	0.227	0.111	0.518	1
	P	0.678	0.794	0.514	0.006	0.001	0.067	0.385	0	0
	n	66	52	59	66	66	66	63	66	66

A: Age of participant at survey, B: Stage of pregnancy, C: Number of people in home

D: PI, E: GI, F: PD, G: LA, H: BANA positive and I: PCR positive.

r = coefficient of correlation, p = p value, n = number of patients.

This table will be used in further points of this study to see the relationship between two variables. Level of significance = $p < 0.05$.

Table 12: Relationship between periodontal indices and stage of pregnancy

	Stage of Pregnancy			TOTAL
	1	2	3	
Number of Patients	1	20	45	66
Mean PI (SD)	1.67	0.40(0.42)	0.38(0.46)	0.40(0.47)
Mean GI (SD)	2.00	0.88(0.59)	1.10(0.49)	1.04(0.54)
Mean PD (SD)	3.67	3.07(0.70)	3.25(0.78)	3.20(0.75)

3.1.6. Association between demographic and clinical variables with clinical indices

Age

Table 11 shows a positive correlation (r = 0.274) between the age at survey and LA (p = 0.030). There was a negative correlation between the ages at survey and PI, PD. The coefficients of correlation (r) were: -0.228 and -0.019 respectively. GI showed no correlation with age.

Number of people in a home

There was a negative correlation between the number of people in a home with PI and GI: -0.221 and -0.146 respectively. There was no significant correlation between the number of people in a home with PD and LA.

History of Pregnancy

Figures 6, 7, 8 demonstrate that the median for PI, GI and PD of women in their first pregnancy (FP) was lower than those who were not in their first pregnancy (MP) while Figure 9 shows no difference in LA between FP and MP.

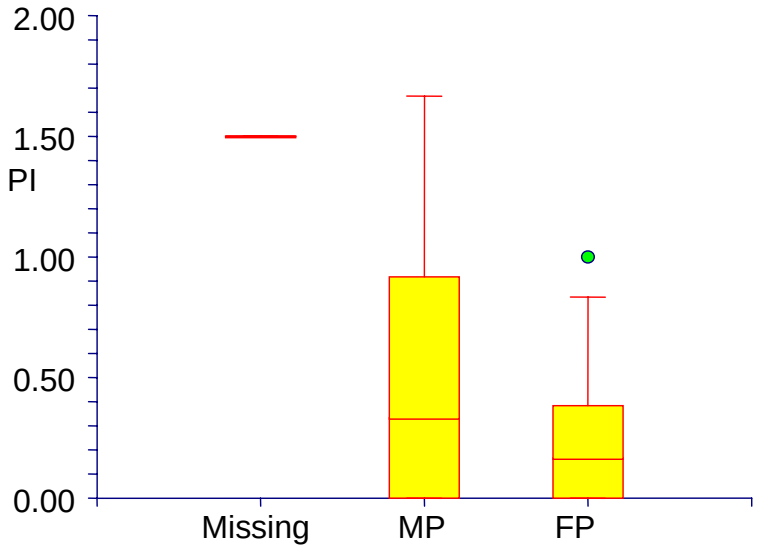

Figure 6: Relationship between first pregnancy or multiple pregnancies and PI

MP: Multiple pregnancies, FP: First pregnancy

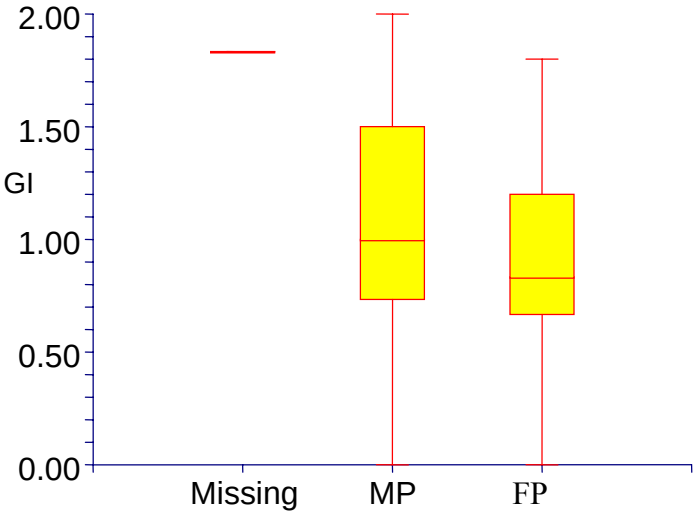

Figure 7: Relationship between first pregnancy or multiple pregnacies and GI

MP: Multiple pregnancies, FP: First pregnancy

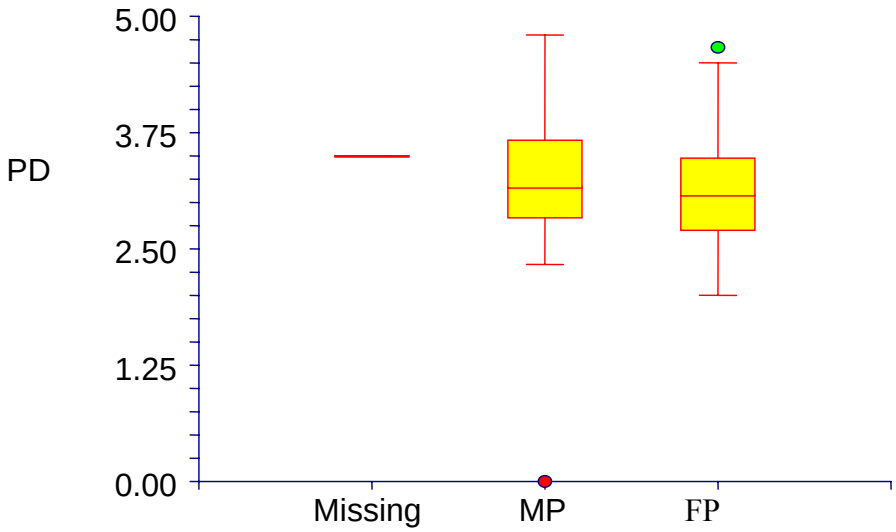

Figure 8: Relationship between first pregnancy or multiple pregnacies and PD

MP: Multiple pregnancies, FP: First pregnancy

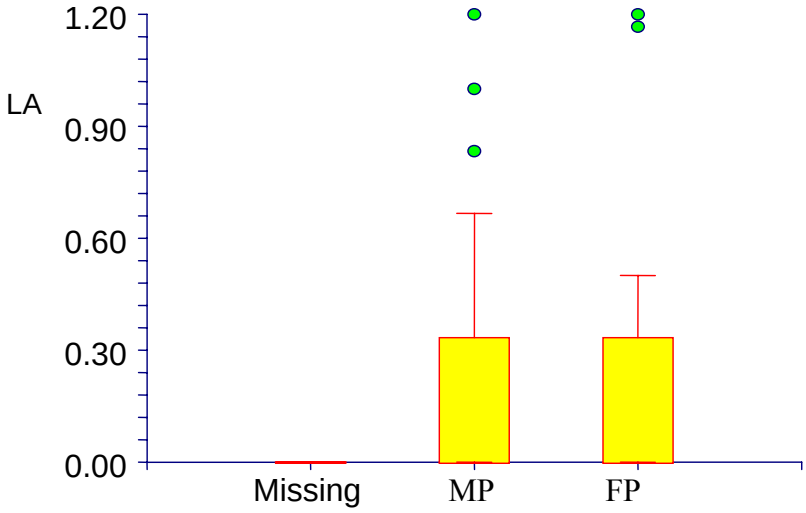

Figure 9: Relationship between first pregnancy or multiple pregnancies and LA

MP: Multiple pregnancies, FP: First pregnancy

HIV- Status

No marked difference was observed between HIV+ and HIV- patients for PI, PD and LA (Figures 10, 12 and 13). The median for GI was lower in HIV-positive patients than in the other groups (Figure 11).

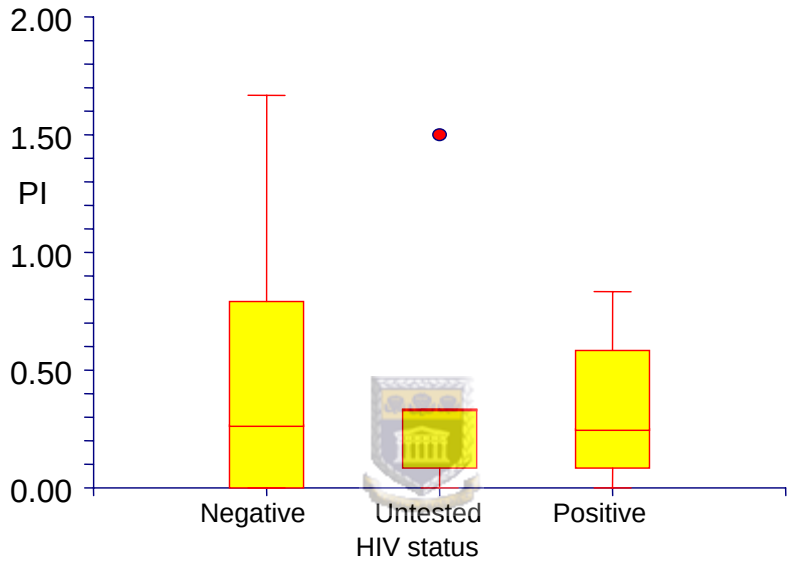

Figure 10: Relationship between HIV status and PI

Cigarette consumption

The median was higher for PI in smokers than in non-smokers (Figures 14) while no differences were observed between smokers and non-smokers for GI, PD and LA (Figures 15, 16 and 17).

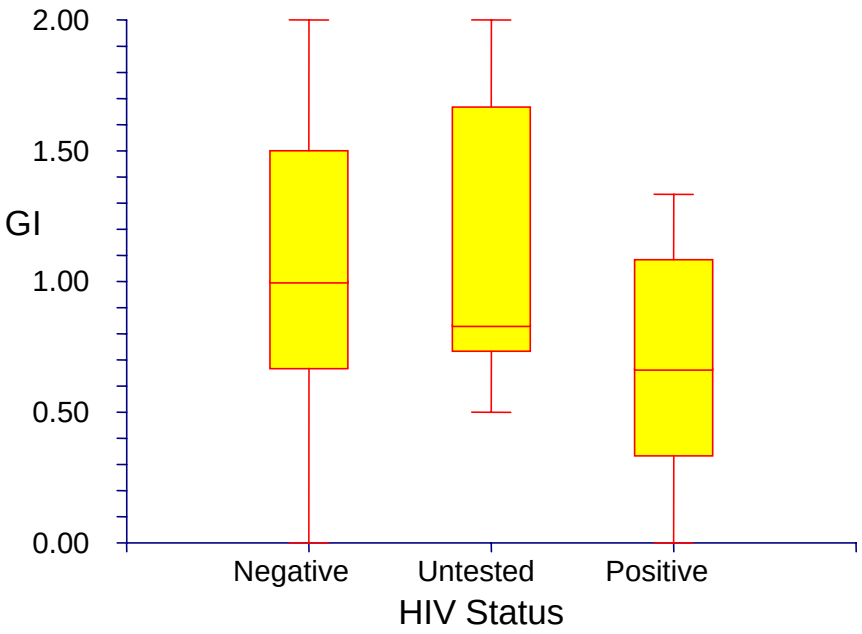

Figure 11: Relationship between HIV status and GI

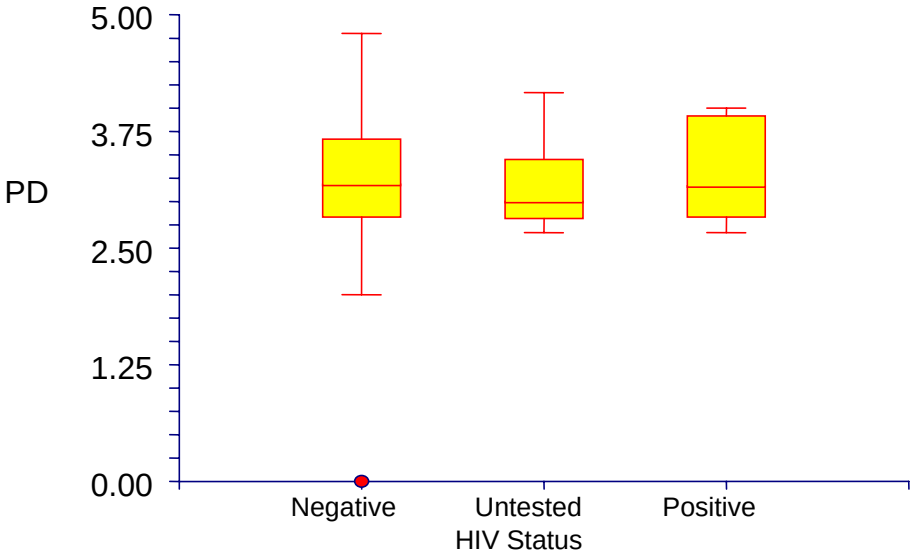

Figure 12: Relationship between HIV status and PD

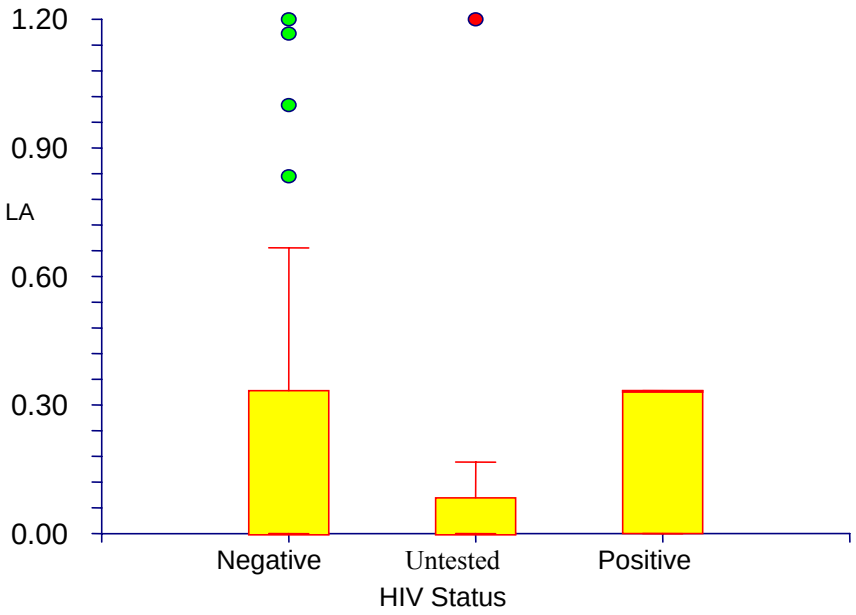

Figure 13: Relationship between HIV status and LA

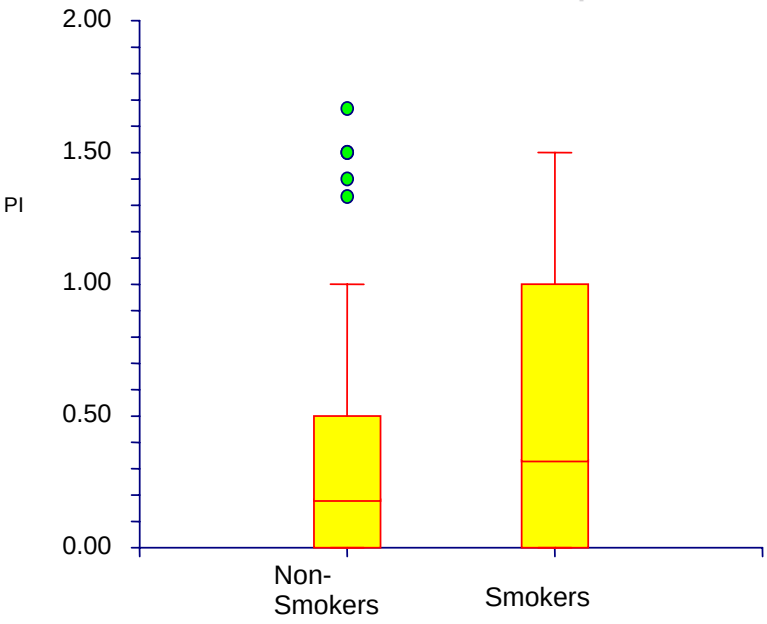

Figure 14: Relationship between smoking and PI

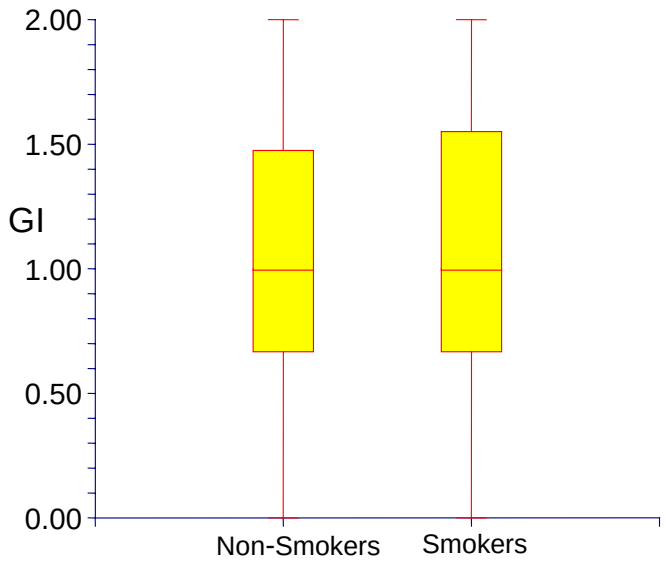

Figure 15: Relationship between smoking and GI

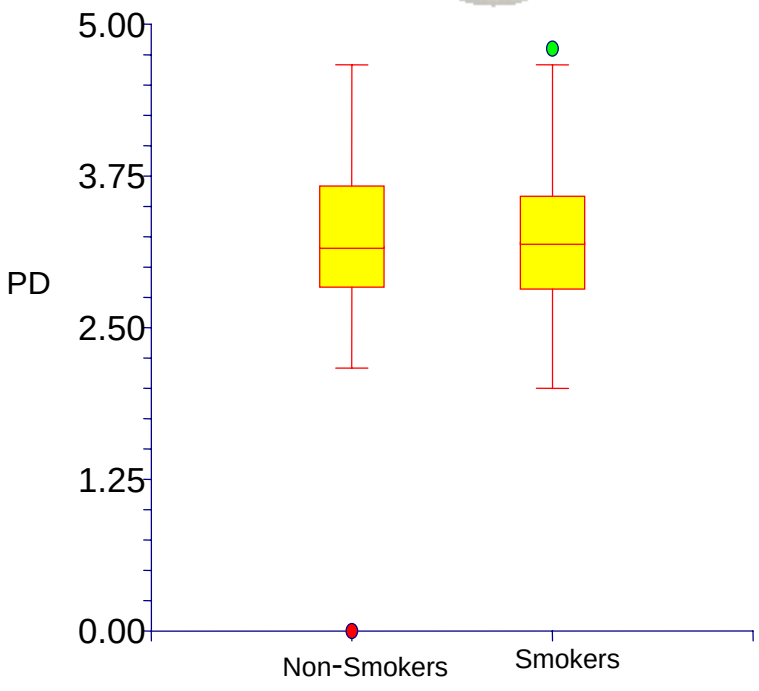

Figure 16: Relationship between smoking and PD

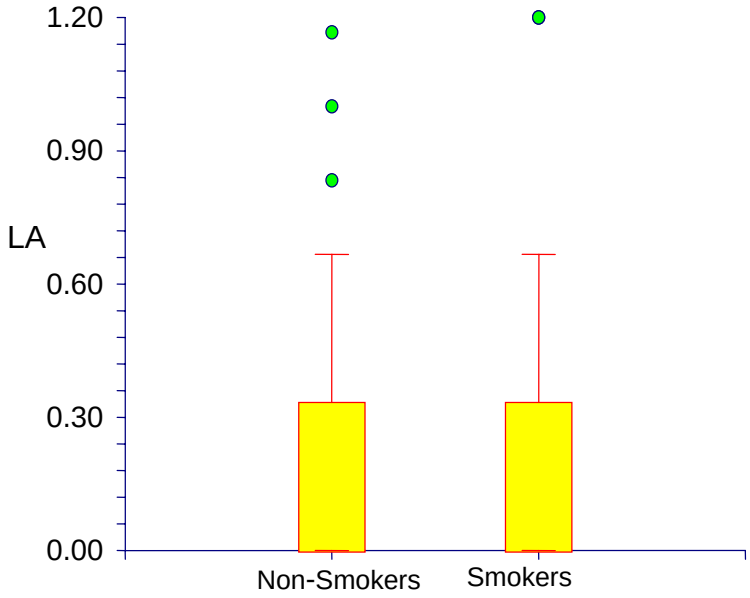

Figure 17: Relationship between smoking and LA

3.1.7. BANA hydrolysis

The BANA test was used to screen for the presence of members of the red complex. Positive and negative BANA reactions are demonstrated in Figure 18. Only 27 (7.25%) of the 372 sites tested positive in 25.75% of the patients examined (Table 13).

Table 13: BANA hydrolysis results

	Patients (n=66)		**Sites (n=372)**	
	n	**%**	**n**	**%**
Negative	49	74.24	345	92.74
Positive	17	25.75	27	7.25

Figure 18: <u>BANA-test card</u>

Left: positive reaction appears as a blue colour to the upper test strip

Right: Negative reaction: no colour

A correlation was sought between BANA, and patient variables (Table 14). Forty-nine of the 66 patients examined tested negative for BANA, 9/66 had 1 BANA-positive site and 7/66 had 2 BANA positive sites. Only one patient had 3 BANA-positive sites. The number of positive BANA sites increased with the stage of pregnancy, and as the number of BANA positive sites increased, so also did the clinical indices. Table 11 shows no significant correlation between BANA and PI, nor between BANA and LA, while GI ($p = 0.030$) and PD ($p = 0.033$) showed a significant correlation with BANA.

Table 14: Correlation between perioscan BANA, clinical indices and stage of pregnancy

	Number of positive BANA sites				TOTAL
	0	1	2	3	
Total number of Patients	49	9	7	1	66
Mean PI (SD)	0.40(0.51)	0.37(0.34)	0.46(0.41)	0.50	0.40(0.47)
Mean GI (SD)	0.97(0.57)	1.09(0.36)	1.40(0.25)	1.67	1.04(0.54)
Mean PD (SD)	3.08(0.74)	3.43(0.79)	3.58(0.49)	4.50	3.20(0.75)
Stage of Pregnancy					
First Trimester	1	0	0	0	1
Second trimester	16	0	3	1	20
Third trimester	32	9	4	0	45

Figure 19 shows the median of BANA positive sites per patient to be the same in women who were in their first pregnancy (FP) and those who had multiple pregnancies (MP). HIV status did not influence the BANA results (Figure 20) while smoking greatly influenced the BANA results. Non-smokers tested negative for BANA while smokers produced more BANA positive sites (Figure 21).

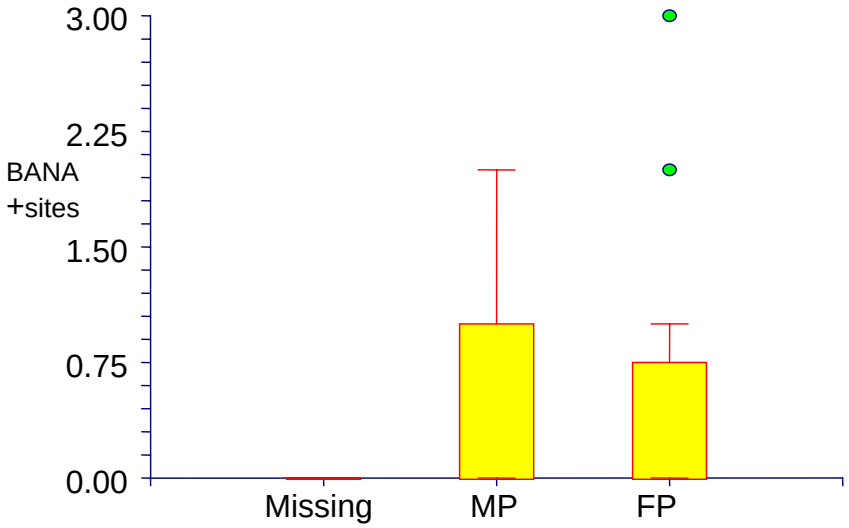

Figure 19: Relationship between first pregnancy or multiple pregnancies and number of BANA positive sites per patient

MP: Multiple pregnancies, FP: First pregnancy

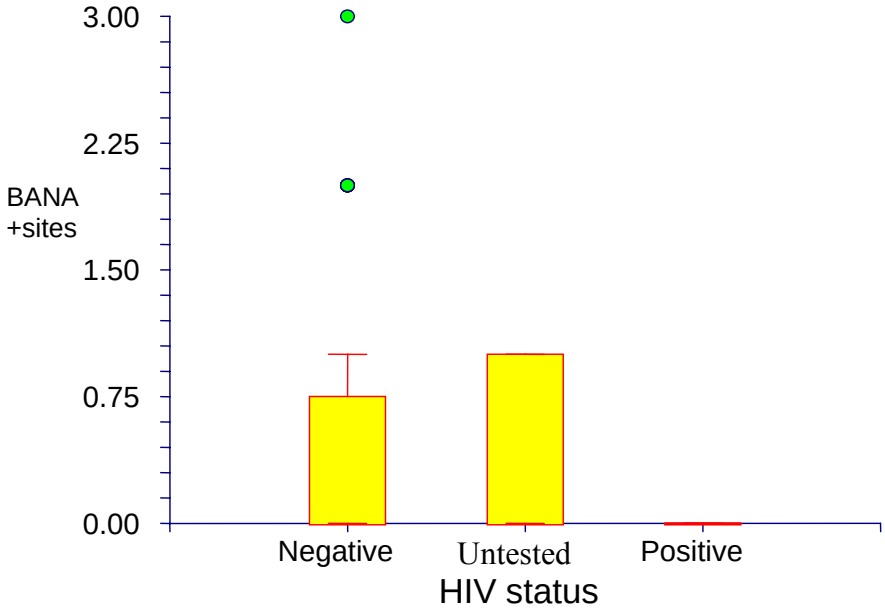

Figure 20: Relationship between HIV status and number of BANA positive sites per patient

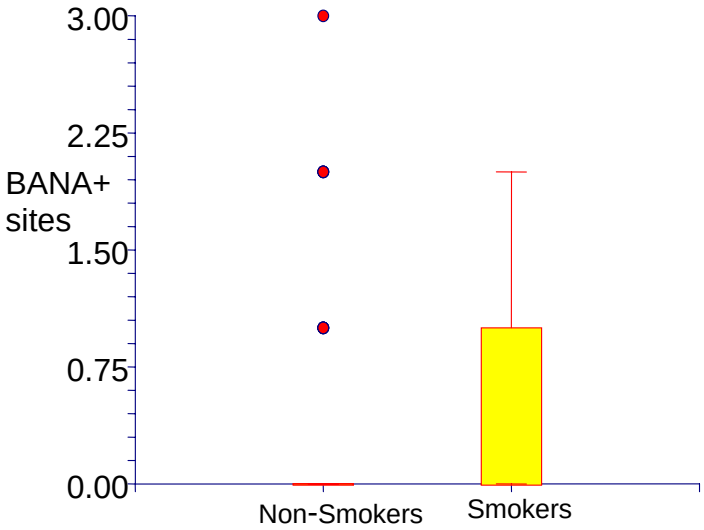

Figure 21: Relationship between smoking and number of BANA positive sites per patient

3.1.8. Prevalence of red complex members detected by PCR

Individual members of the **red complex** were tested for using PCR. Examples of PCR detection of *T. denticola, T. forsythensis, P. gingivalis* respectively are demonstrated in Figures 22-24. Table 15 reports on the prevalence of the **red complex** in the subject group while Table 16 reports on the prevalence of the **red complex** in sites from the subject group. Of the subjects examined, 74.2% tested positive by PCR for at least one member of the red complex (Table 15), with a total of 36.29% of the sample sites yielding a positive BANA test (Table 16). *P. gingivalis* was the most frequently detected, followed by *T. forsythensis* and lastly *T. denticola* (Table 15). All three members of the red complex were detected in 16.6% of the subjects while 28.7% harboured species in pairs with their prevalence occurring in order of *T. forsythensis/P. gingivalis* (18%), *P. gingivalis/T. denticola* (6%) and *T. forsythensis/T. denticola* (4%).

Table 15: Prevalence of red complex members among subject group

PCR Results	Patients	
	n = 66	**%**
P.gingivalis	10	15.15 %
T. forsythensis	6	9.09 %
T.denticola	3	4.54 %
T. forsythensis/P.gingivalis	12	18.18 %
P.gingivalis/ T.denticola	4	6.06 %
T.forsythensis/T.denticola	3	4.54 %
Red complex (all 3 species)	11	16.66 %
Negative	17	25.8 %
Total of Positive	49	74.2 %

A similar trend is apparent when we examine the prevalence of these species in the different sites (Table 16). Appendix 3B gives the breakdown of PCR results in different Ramfjord teeth.

Table 16: Prevalence of red complex members according to sites

PCR Results	Sites	
	n = 372	**%**
P.gingivalis	55	14.78
T. forsythensis	21	5.64
T.denticola	8	2.15
T. forsythensis/P.gingivalis	23	6.18
T.forsythensis/T.denticola	10	2.68
P.gingivalis/ T.denticola	9	2.42
Red complex (all 3 species)	9	2.42
Negative	237	63.70
Total of Positive	135	36.29

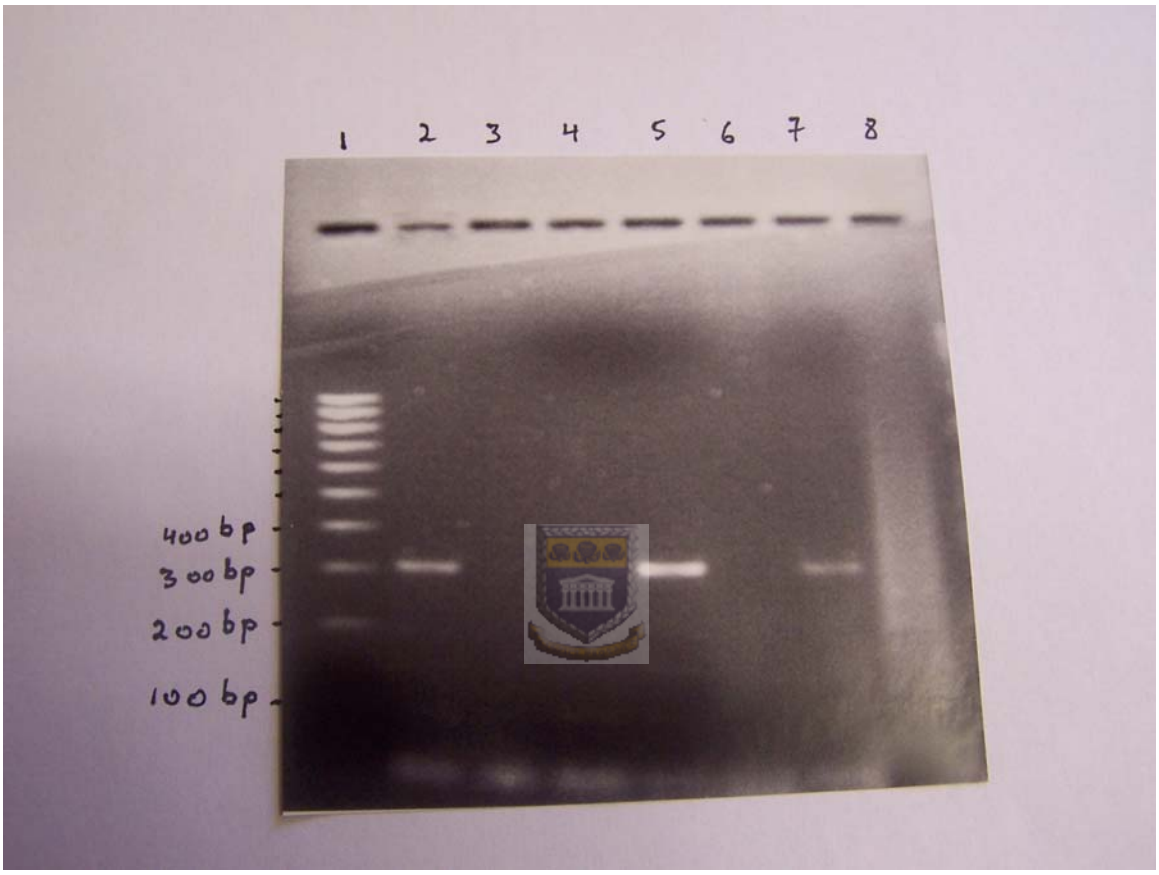

Figure 22: PCR amplification of *T. denticola* using Species-specific primers

Expected product size: 316 bp, lane 1: DNA marker 100 bp, lane 2: positive control, lane 3: negative control, lanes 4, 5, 7: samples showing target DNA amplifications, lanes 6, 8: no amplification.

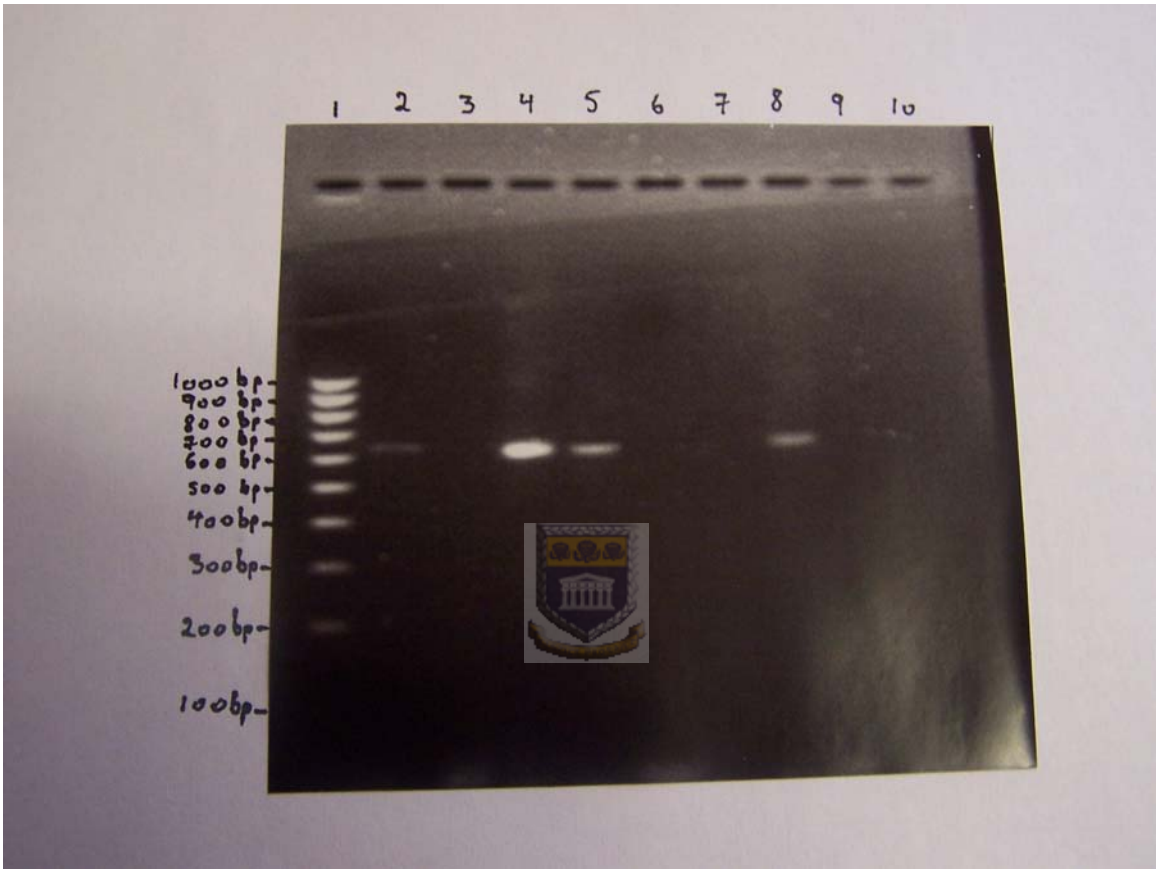

Figure 23: PCR amplification of *T. forsythensis* using Species-specific primers

Expected product size: 641 bp, lane 1: DNA marker 100 bp, lane 2: positive control, lane 3: negative control, lanes 4, 5, 8: samples showing target DNA amplifications, lanes 6, 7, 9, 10: no amplification.

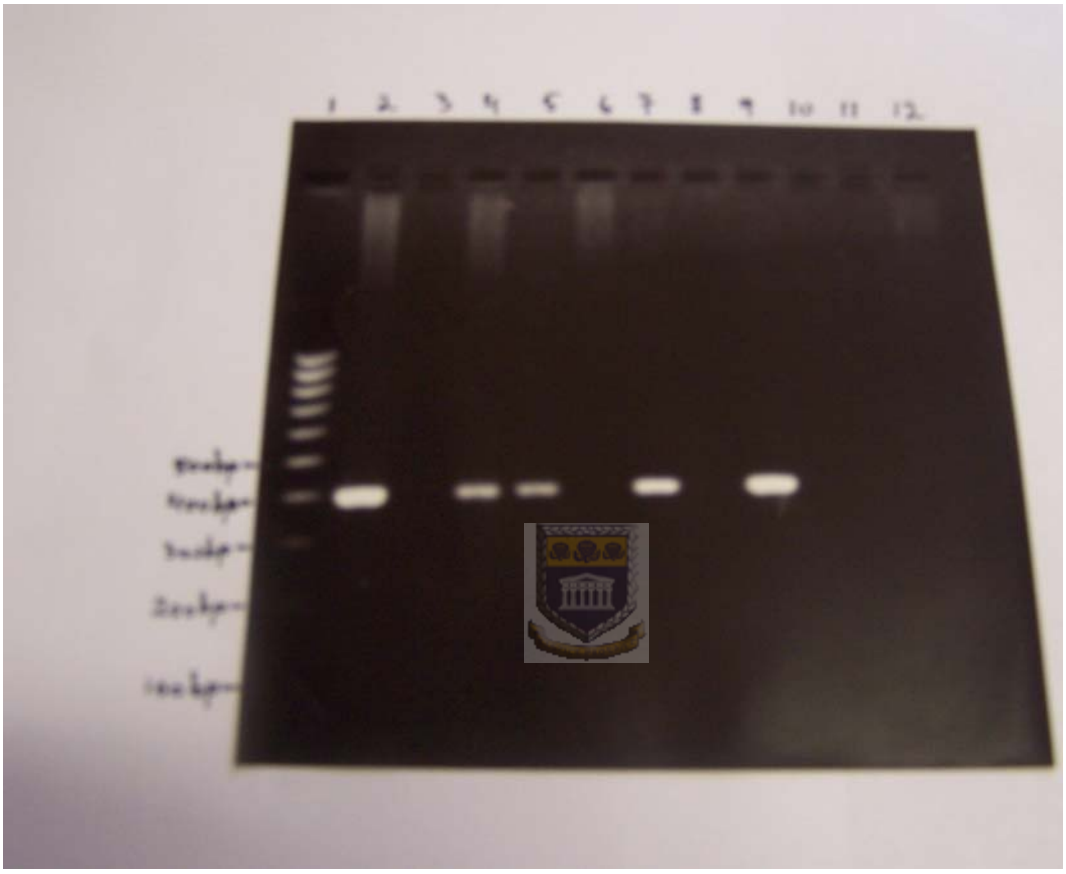

Figure 24: PCR amplification of *P. gingivalis* Using Species-specific primers

Expected product size: 404 bp, lane 1: DNA marker 100 bp, lane 2: positive control, lane 3: negative control, lanes 4, 5, 7, 9: samples showing target DNA amplifications, lanes 6, 8, 10, 11, 12: no amplification.

3.1.9. Associaton between red complex members (as detected by PCR) and clinical variables

The mean PI for subjects with no positive sites was 0.25 (±0.41) while patients with 1-2 positive sites and those with 3 and more positive sites had means of 0.4 (±0.49) and 0.5 (±0.47) respectively (Figure 25). These results correspond with Table 11 where a significant correlation (r = 0.336) between PCR-positive results and PI (p = 0.006), and PCR- positive results and GI (r = 0.396, p = 0.001) are reported. No significant correlation (r = 0.227, p = 0.067) was observed between PCR-positive results and PD (Table 11 and Figure 25).

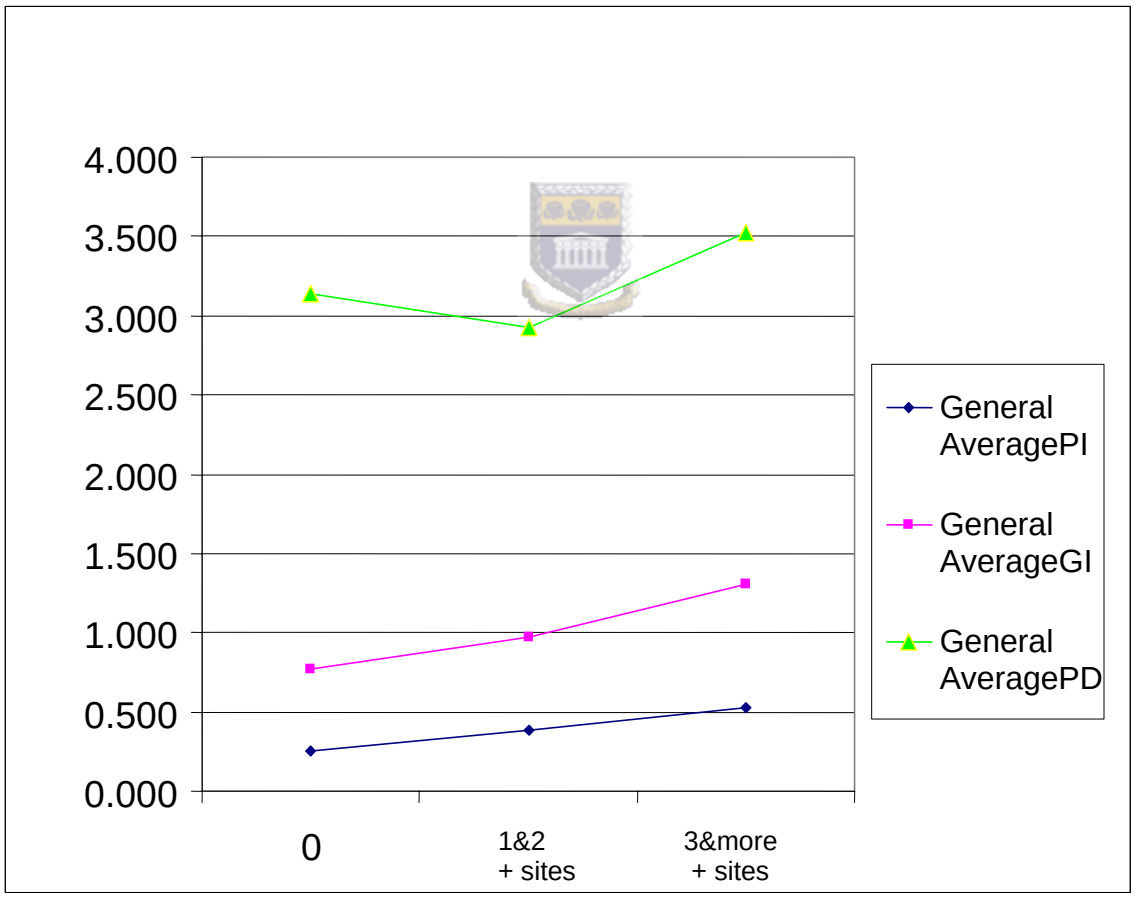

Figure 25: Red complex members and clinical indices

Figure 26 shows that no difference was observed between the median of PCR positive sites per patient for women who were in their first pregnancy (FP) and those who had multiple pregnancies (MP), neither did the HIV+ subjects show any association with PCR detection (Figure 27). More PCR positive sites were observed in smokers than non-smokers (Figure 28).

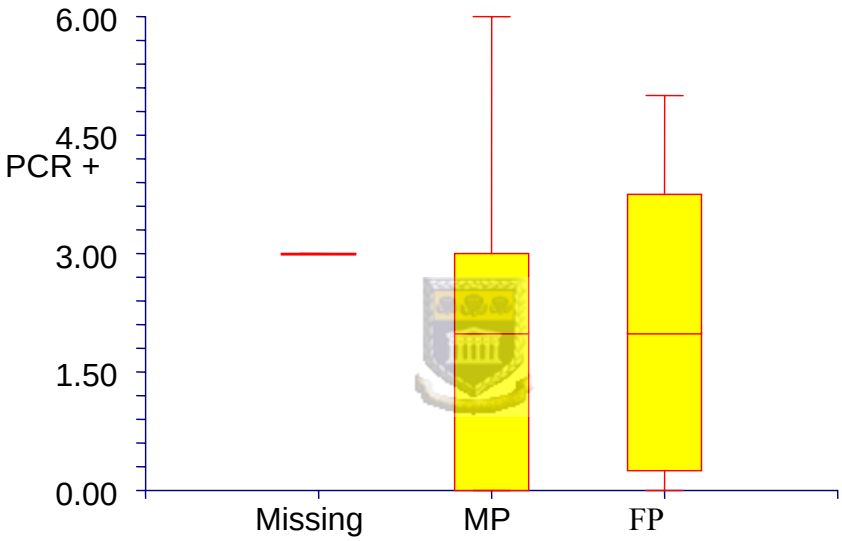

Figure 26: <u>Relationship between first pregnancy or multiple pregnancies and number of PCR positive sites per patient</u>

MP: Multiple pregnancies, FP: First pregnancy

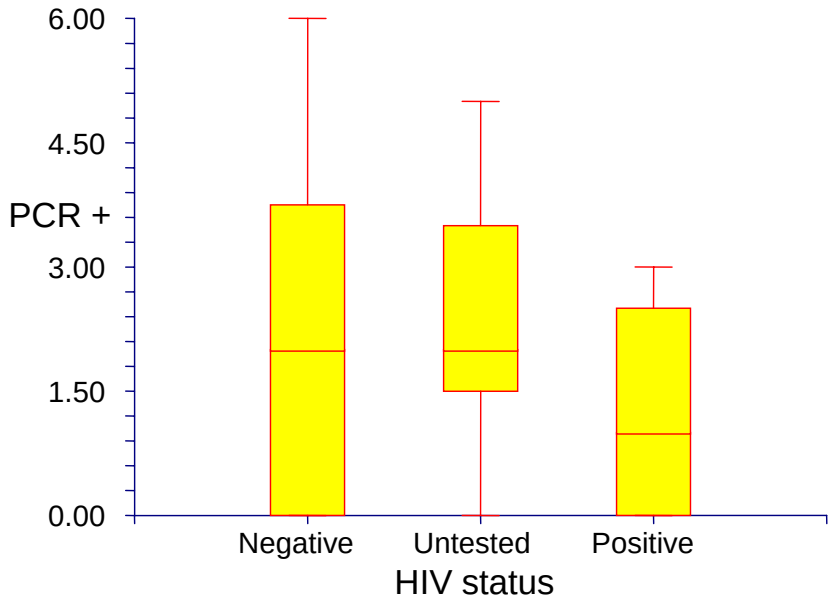

Figure 27: Relationship between HIV status and number of PCR positive sites per patient

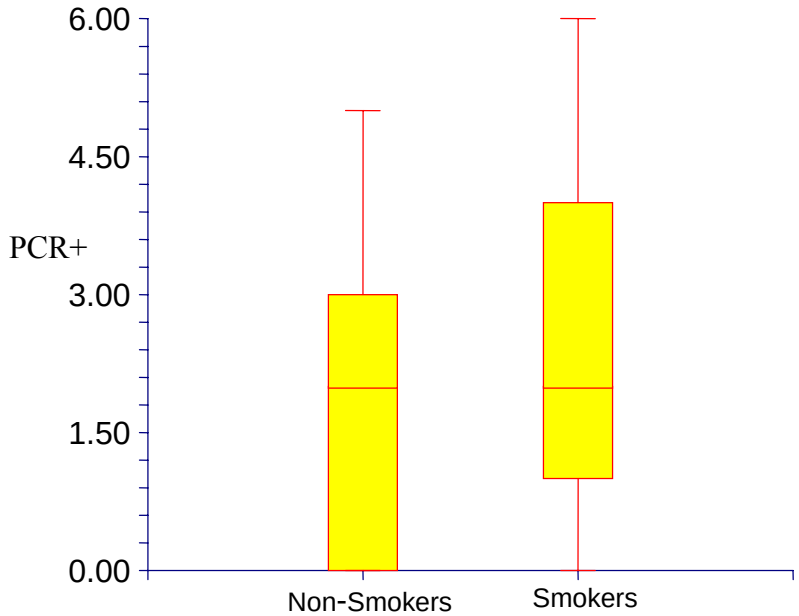

Figure 28: Relationship between smoking and number of PCR positive sites per patient

Earlier an association between BANA and stage of pregnancy was demonstrated. Using PCR, it was possible to define exactly which member of the **red complex** contributed to the BANA reaction in a particular site. When looking for an association between members of the **red complex** and stage of pregnancy (Table 17), it was observed that *P. gingivalis* had the greatest prevalence, followed by *T. forsythensis* with *T. denticola* only appearing in the 3rd trimester. No significant increase was noted between the 2nd and 3rd trimester for *T. forsythensis/P. gingivalis*, nor *P. gingivalis/T. denticola*, however, a significant increase was observed in the 3rd trimester for *T. forsythensis/T.denticola* .

Fifteen of the 20 patients (Table 18) who presented in the 2nd trimester of pregnancy were PCR positive (75%) while only 33/45 (73%) in the 3rd trimester were PCR positive. Only one patient tested positive for all six sites and she was in the 2nd trimester of her pregnancy.

Table 17: Members of red complex and stage of pregnancy

	2nd Trimester	**3rd Trimester**
***P.gingivalis* n(%)**	17(38.63 %)	38(41.75 %)
***T. forsythensis* n(%)**	8(18.18 %)	13(14.28 %)
***T.denticola n*(%)**	0	8(8.79 %)
***T.forsythensis/P.gingivalis* n(%)**	11(25 %)	12(13.18 %)
***P.gingivalis/ T.denticola* n(%)**	4(9.09 %)	5(5.49 %)
***T.forsythensis/T.denticola* n(%)**	1(2.27 %)	9(9.89 %)
Red complex n(%)	3 (6.81 %)	6(6.59 %)

Table 18: Number of PCR positive sites and stage of pregnancy

Stage of pregnancy	Number of positive PCR sites							TOTAL
	0	1	2	3	4	5	6	
1st Trimester	0	0	1	0	0	0	0	1
2nd Trimester	5	4	4	1	3	2	1	20
3rd Trimester	12	6	10	8	5	4	0	45
TOTAL	17	10	15	9	8	6	1	66

3.1.10. Comparison of BANA hydrolysis and the presence of red complex members detected by PCR

Table 19 shows the comparison of BANA hydrolysis and PCR in detecting the **red complex**. Overall, more sites tested positive with PCR than with BANA. In order to determine the accuracies of the two detection methods, sensitivity, specificity, % accuracy, positive and negative predictive values and odds ratios were calculated.

When using BANA as the reference standard, and PCR as the test a sensitivity of 96.42% and a specificity of 68.6% was obtained, along with an accuracy of 70.69% and positive and negative predictive values of 20% and 99.57% respectively (Table 20).

When PCR was used as the reference standard, the sensitivity of BANA in detecting members of the **red complex** dropped to 20% while specificity and PPV increased to 99.57% and 96.4% respectively. BANA showed excellent specificity and positive predictive values for all three members of the **red complex** whether they occurred singly

or in pairs (Table 21). The accuracy of 64-66% was influenced by the specificity (100 %) since the sensitivity was rather low. The results in Tables 20 and 21 show that PCR was more sensitive in detecting the **red complex** than BANA.

The above results indicate that although sensitivity, specificity and accuracy values provide a good indication of the validity of the test procedure, they do not necessarily predict the outcome if the result is unknown. A better representation of the validity of the test procedure is indicated in the odds ratios, as reflected in Tables 20 and 21.

Table 19: Relationship between BANA hydrolysis in different sites and the presence of red complex members detected by PCR

	Number of PCR + Sites	BANA Positive		BANA Negative	
		n	%	n	%
P.gingivalis	55	11	40.74	44	12.75
T. forsythensis	21	1	3.70	20	5.79
T.denticola	8	1	3.70	7	2.02
T.forsythensis/P.gingivalis	23	7	25.92	16	4.63
T.forsythensis/T.denticola	10	0	0	10	2.89
P.gingivalis/ T.denticola	9	4	14.81	5	1.44
Red complex (All 3)	9	2	7.40	7	2.02
Negative	237	1	3.70	236	68.40

Table 20: Comparison of BANA (reference) results and PCR

Reference %	Sens.	Spec.	Acc.	PPV	NPV	Odds +	Odds -
PCR	96.42	68.60	70.69	20	99.57	3.09	0.03

Sens.: sensitivity, Spec.: specificity, Acc.: Percentage accuracy, PPV: positive predictive value, NPV: negative predictive value, Odds +: positive odd ratio, Odds -: negative odd ratio.

Table 21: Comparison of PCR (reference) results and BANA

Reference %	Sens.	Spec.	Acc.	PPV	NPV	Odds+	Odds -
BANA	20	99.57	70.69	96.42	68.60	50	0.01
P.gingivalis	20	100	66.66	100	84.34	-	0.80
T.denticola	12.5	100	63.97	100	97.13	-	0.87
T.forsythensis	4.76	100	63.97	100	92.21	-	0.95
P.gingivalis/T.denticola	44.44	100	64.78	100	97.93	-	0.55
T.forsythensis/P.gingivalis	30.43	100	64.51	100	93.67	-	0.69
T.forsythensis/T.denticola	0	100	63.70	100	95.95	-	1
Red complex (all 3*)*	22.22	100	64.24	100	97.13	-	0.77

CHAPTER IV: DISCUSSION AND CONCLUSION

The objective of the study was to establish the prevalence of the **red complex** in women attending a MOU in order to determine their risk of preterm or low birth weight delivery as a consequence of periodontal disease.

The level of education, living conditions and diet may all contribute to the general health of a mother. Proper hygiene habits, weight control and regular visits to the dentist and gynecologist should ensure that a woman maintains good health throughout her pregnancy and delivers a healthy infant. Obstetric risk factors were assessed by means of a questionnaire. Half of the study group were young, aged between 17 and 25 years, and uninformed. Many were not aware that their oral status may impact on their infants. Most of the subjects (68.8%) visited the clinic for the first time in their third trimester of pregnancy. A DMF-T score of > 7 was recorded for >67% of the subjects of whom 40-47% admitted visiting a dentist or gynecologist only when necessary. A large percentage (93%) reported having no medical insurance and this could account for their lack of annual medical and dental check-ups. Mitchells Plain is an area in which most people live below the breadline thus limiting effective nutrition and health care.

Measurement of the clinical indices showed that 68.5% of the 372 sites measured had PI = 0, and the mean (SD) PI for all the teeth was 0.4 (±0.47). Silness and Löe (1964) found a mean PI of 0.85 ± 0.024. Tilakaratne *et al*. (2000) reported a mean PI of 0.69 at the third trimester of pregnancy. The low PI of our study population may be due to better oral hygiene practices than these previous studies and the time of the day when the samples were collected. All the samples were collected in the early morning just after tooth brushing and before plaque was allowed to build up. There was no significant difference between the mean PI of women in the second (0.40 ± 042) trimester and those enrolled in the third trimester (0.38 ± 0.46). These results disagree with those reported in previous studies (Silness and Löe, 1964; Yalcin *et al*., 2002; Taani *et al*., 2003) but agree with the one reported by Tilakaratne *et al*., (2000).

Earlier studies showed the prevalence of pregnancy gingivitis ranging from 35% to 100 % (Miyakazi *et al*., 1991; Rossel *et al*., 1999; Yalcin *et al*., 2002; Family Gentle Dental Care, 2003). GI=1 indicates a mild inflammation with a slight change in colour and texture of the gingivae. According to the criteria for the GI, this study showed that 78.3% of pregnant women showed signs of gingival inflammation with GI=1 in 54.9% and GI=2 in 22.9%. The mean GI was 1.04 (±0.54) which was similar to results found by Löe and Silness (1963) in their study of pregnant women (1.03 ± 0.03). Lieff *et al*.(2004) in their study on oral conditions and pregnancy, found that 28.1% of pregnant women had a score of 0 and 71.9% of them had a score of GI ≥ 1. The influence of the stage of pregnancy on gingival scores was examined and it was found that the mean GI increased from 0.88 ± 0.59 to 1.10 ± 0.49 from second to third trimester. Similar results were reported by Löe and Silness, (1963), Nuamah and Annan, (1998), Agbelusi *et al*., (2000), Tilakaratne *et al*., (2000), Yalci *et al*., (2002) and Taani *et al*., (2003). It is believed that in the third trimester, progesterone and estrogen levels increase 10 to 30 times greater than during the menstrual cycle (Zachariasen *et al*., 1993). This increase causes dilatation of gingival capillaries, gingival exudates and permeability which can demonstrate the redness and bleeding tendency during pregnancy (Sooriyamoorthy, 1989).

In this study we found an increase in GI and PD scores from the second trimester to the third trimester, but no significant difference in PI and LA was observed between second and third trimesters. The mean pocket depth of our study population was 3.2 (±0.75) mm. Sixty-six percent of the 372 sites measured had a PD between 1 and 3mm and 33.1% had a PD between 4 and 6mm. Löe and Silness, (1963) found a mean PD of 3.2 ± 0.05. Moore *et al.*, (2001), in their study on periodontal health of London women during early pregnancy, found a mean PD of 2 ± 0.4 while Lief *et al*., (2004) reported a mean PD of 1.6 ± 0.5. The higher mean of PD in our population study may be due to the higher frequency of pregnant women at the third trimester (68.8 %). The mean PD of patients enrolled in the second trimester increased from 3.07 ± 0.70 to 3.25 ± 0.78 for those enrolled at their third trimester. Similar results were found by others (Löe and

Silness, 1963; Agbelusi *et al*., 2000; Tilakaratne *et al*., 2000; Yalcin *et al*., 2002; Taani *et al*., 2003).

There was a positive correlation between the increase of age at survey with LA but not with PI and PD. Yalcin *et al*., (2002) reported no association between the increase of age and clinical parameters. These findings disagree with Taani *et al*., (2003) who reported that the increase of age was associated with significantly higher GI and PD scores.

Women who had previous or multiple pregnancies had higher PI, GI and PD scores than women in their first pregnancy. These results support the findings of Taani *et al*., (2003) who found women with previous or multiple pregnancies had statistically significant higher GI and PD scores than women in their first pregnancy.

A high prevalence of HIV-associated periodontal disease has been reported (Masouredis *et al*. 1992, The America Academy of Periontology, 1994, Schuman *et al*., 1998, Scully *et al.,* 1999, Robinson *et al*., 2002). In the present study, HIV did not remarkably alter the clinical indices of the patients examined, unlike the study of Ndiaye *et al*. (1997) who reported higher scores of GI and PD in seropositive than seronegtive subjects. Gornitsky *et al*., (1991) found that the mean PI and PD were similar for seropositive and seronegative individuals as in our study.

As in the study by Moore *et al*. (2001), smokers in this study demonstrated higher median PI scores with more BANA and PCR positive sites per patient than the non-smokers. Loesche *et al*., (1997a), Zambon *et al*. (1996), and Kazor *et al*. (1999) reported that smokers are 10 times more likely to have BANA positive plaques than non smokers, which is what we found in this study. Although smoking has been reported to contribute to the colonisation of suspected periodontopathogens in this and the above studies, the reasons for this have not been elucidated and requires further investigation (Shiloach *et al*., 2000).

Twenty-five point seventy-five percent of the population group showed a positive BANA-test but only 27 (7.25 %) of the 372 sites were positive. Of the 6 sites examined in each of the 66 patients, it was found that all patients had no more than 3 sites which tested positive for BANA. The use of 6 Ramfjord teeth instead of 2 or 3 random sites therefore confirms the need for examination of more sites. The use of six Ramfjord teeth is useful in predicting the whole mouth periodontal status (Gettinger *et al*., 1982; Silness and Roynstrand, 1988; Baelum *et al*., 1995; Mumghamba *et al*., 2004), and reduces the risk of missing out sites which may be breaking down.

There was no significant correlation between BANA and PI, while a positive correlation was demonstrated between BANA and GI, and between BANA and PD. These findings suggest a correlation between BANA and the clinical indices used to indicate periodontal infection with the **red complex**. Similar results were found by others in previous studies (Loesche *et al.,* 1990; Grisi *et al*., 1999; Grisi *et al*., 2001; Figueiredo *et al*., 2000). The number of BANA positive sites increased from the 2nd to 3rd trimester of pregnancy, and in women who had previous pregnancies.

The PCR results showed at least one of the members of the **red complex** occurred in 74.2% of women and of the 372 samples collected, only 36.29% of sites were PCR-positive. This result demonstrates once again the importance of the use of the six Ramford teeth instead of one or two random sites. Many patients (18.18%) harboured the pair *T.forsythensis/P.gingivalis*. Previous studies have reported the synergy between *T.forsythensis* and *P.gingivalis* (Zambon, 1996; Takemoto *et al*., 1997; Klein and Goncalves, 2003), with a decrease in frequency after therapy (Takamatsu, 1997). All three members of the **red complex** occurred in 16.66% of women. *P.gingivalis* was prevalent in 15.15%, *T. forsythensis* occurred in 9.09% with *T. denticola* occurring in only 4.54% of patients. Previous studies found *T.forsythensis* more prevalent than *P.gingivalis* (Klein and Goncalves, 2003; Takamatsu, 1997) while other studies found *P.gingivalis* to be more prevalent than *T. forsythensis* (Kasuga *et al*., 2000; Lee *et al*., 2003).

There was a positive correlation found between PCR and PI, and between PCR and GI. This correlation between **red complex** members and clinical indices has been reported previously (Kasuga *et al*., 2000, Tanaka *et al*., 2002, Klein and Goncalve, 2003). No significant correlation was found between PCR and PD.

PCR-results showed a good agreement with BANA when BANA was used as the reference standard. The weak PPV (20%) was due to the few positive BANA results compared to the positive PCR-results. Only one site in this study tested positive for BANA and negative for PCR. This could account for the sensitivity of 96.4% recorded in Table 20 instead of 100%. Because BANA is unable to specify which species is present, we had to rely on PCR to establish the prevalence of each of the members of the **red complex.**

When PCR was used as the reference standard, BANA results showed good specificity, percentage accuracy, positive and negative predictive values, but the sensitivity of the test was very low (20%). Table 11 showed a positive correlation (r = 0.518) between BANA and PCR (p = 0.000). Previous studies showed that BANA was very sensitive (Loesche *et al*., 1990, 1991, 1992, 1997), but another study found a specificity of only 61% and a sensitivity of 41.7% for BANA (Neuenschwander *et al*., 1992). These studies examined diseased sites only, while our study examined random sites whether or not they were diseased. Comparison of BANA with ELISA for detecting *P.gingivalis*, *T.denticola* and *T.forsythensis* was 85 %, 75% and 65% accurate respectively; and 54%, 89% and 34% accurate when compared with culture (Loesche *et al*., 1992). Loesche *et al*. (1997) reported a frequency of 9% of false-positive BANA. *Bacteroides* and *Capnocytophaga* species were reported to have a weak BANA-positive reaction (Loesche *et al*., 1990).

When *P.gingivalis* occurred on its own the sensitivity was 20% and this increased to 22.22% when all 3 members of the **red complex** occurred together, showing the greatest sensitivity when combined with either *T.denticola* (44.4%) or *T.forsythensis* (30.4%). Loesche *et al*., 1990, found that the perioscan results were 85 % sensitive, 53 % specific

and 79 % accurate related to the presence or absence of either *T.denticola* or *P.gingivalis* or both in plaque samples. Although the sensitivity was lower in the present study, the PPV for each of the species, either on its own or in association with other members, was 100 %.

The PCR results showed that 74.2% of the subjects harboured at least one member of the **red complex**. *P.gingivalis* was the most prevalent of the three members of the **red complex**. PCR, therefore appears to be a more sensitive detection method for the **red complex** than BANA. PCR has a high level of sensitivity and has been used successfully for the identification of periodontal pathogens (Klein and Gonsalve, 2003, Watanabe and Frommel, 1993, 1996, Tran and Rudney, 1996, 1999, Lee *et al*, 2003, Kasuga *et al*, 2000, Conrads *et al*, 1999).

The present study revealed a positive correlation between BANA and PCR. No significant association was observed between PCR and PD or LA although there was a correlation with PI and GI. A significant correlation was also found between BANA and PD as well as GI.

Findings of this study confirm the need for oral hygiene instruction and dental preventive measures in pregnant women. Although the sample number of 66 may be considered small, we considered the number of 372 sites adequate for the comparison of BANA and PCR. Microbial monitoring of potential colonization of periodontopathogens may play a major role in preventing periodontal disease associated with pregnancy, thus reducing the risk for LBW or pre-term infants. While it can't be disputed that PCR is a more sensitive detection method for members of the **red complex**, there is the risk of very small numbers of bacteria being detected even in sites which are considered to be healthy, as reported by Wilson *et al*., (1993), Lee *et al*., (2003) and Klein and Gonçalves (2003). One can therefore conclude that although PCR is the better test for detecting small numbers of members of the **red complex** in plaque samples, BANA is a more sensitive indicator of gingivitis backed by the significant correlation with PD and GI which are the clinical indices used in the diagnosis of gingivitis. Since 78.3% of

pregnant women in this study showed signs of gingival inflammation (GI ≥ 1), the above results suggest that BANA may therefore be a better predictor of mothers at risk for adverse pregnancy outcomes. Follow-up studies are currently underway to establish whether any of the mothers in present study who, according to the BANA test, appeared to be at risk for periodontal disease, delivered pre-term or low birth weight infants. The outcome of these studies may pave the way for effective identification of mothers at risk, thus reducing the risk of infant mortality as a result of pre-term or low birth weight delivery.

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APPENDIX 2A

CONSENT FORM FOR PARTICIPATION IN RESEARCH PROJECT

Title of Project: BANA screening for periodontal disease in a sample of HIV positive and HIV negative pregnant women in the Western Cape.
Names of Researchers: Prof Charlene WJ Africa, Dr Soraya Harnekar, Mr Claude Bayingana (MSc student)

If you would like to participate in this study please tick the relevant boxes:

1. Have you read the attached information sheet and has the purpose of the research project been explained to you? Yes or No

2. Do you understand the method of sample collection and any risks involved? Yes or No

3. Do you grant permission for information from your medical records to be disclosed to the research team as and when necessary? Yes or No

Do you agree that samples collected for research or diagnostic testing can be stored for possible use in future research projects conducted by the above named researchers and /or other research collaborators? Yes or No

I declare that my participation in this research project is voluntary and that I am free to withdraw my approval for use of the sample(s) at any time without giving a reason and without my medical treatment or legal rights being affected. I understand that any information contained in my file will remain confidential and that I (or my doctor) will be informed if any of the results of the medical tests done (as part of the research) have implications for my health. I know how to contact members of the research team should I change my mind about participating in this study.

……	…….	……
Name of patient (BLOCK CAPITALS)	**Date**	**Signature**
……	……	……
Name of person taking consent	**Date**	**Signature**
……	……	……
Name of researcher	**Date**	**Signature**

THANK YOU FOR AGREEING TO PARTICIPATE IN THIS RESEARCH

APPENDIX 2B

INFORMATION SHEET

Prospective participants are requested to read this information sheet carefully and to ask questions where necessary, before signing the attached consent form. This sheet must be detached and retained by the participant and the consent form filed for record.

The purpose of the research project is to compare the bacterial species in the mouths of pregnant women in order to establish whether the prevalence of certain species can be used as indicators for pre-term delivery or delivery of babies with low birth weights. The effect of HIV infection on pregnancy gingivitis has thus far not been documented and the outcome of this study will therefore provide valuable information regarding the impact of the oral health of a HIV-positive mother on her infant.

The clinical procedure will entail the measurement of oral clinical indices for use in making a clinical diagnosis of pregnancy gingivitis and the removal of plaque samples from selected teeth for laboratory investigation. The clinical measurements and sample collection are non-invasive and safe and will be carried out with the utmost care to ensure the comfort of the patient.

It must be brought to the attention of the individual that pregnancy gingivitis may present with bleeding gums and that any bleeding during the procedure may therefore be due to this condition (as during tooth brushing) and not due to injury inflicted by the dental examiner. The bleeding will be of short duration and will pose no major threat or discomfort to the individual. If the gums are healthy, no bleeding will occur.

Patients will be required to sign the attached form granting consent for these procedures to be carried out and for the subsequent use of the samples donated and clinical parameters recorded. The patient will also be required to grant permission for her HIV status and other medical history to be disclosed. Participants will not be recorded by name, but samples and information will be coded to protect the identity of the individual.

However, the coding will be used by the clinic to trace the individual if relevant information (as a result of the study) should be passed to her or her doctor. Where necessary, participants with severe periodontal disease will be referred for treatment. Permission will also be sought for the use of additional biological material collected in the clinic, which is usually discarded but which the researchers may find useful for future research.

Participation in this study is voluntary and refusal to participate will not prejudice the treatment of the patient in any way. Consent to participate will be recorded by completing the attached form. Should individuals agree to participate and later change their minds, they may withdraw by calling the following persons:
Prof C. Africa, University of the Western Cape, Department of Medical Biosciences, Tel: 021 9592342, or Dr S Harnekar, Oral Health Centre, Mitchells Plain, Tel: 021 3704400.

APPENDIX 2C

Health Risk Assessment Questionnaire

Dear patient,

As part of this study we need to collect information pertaining to your lifestyle so that an assessment of your health risks may be made. Any information contained on this sheet will be held in the strictest confidence and I would urge you to respond to the questions with accuracy. No details of personal identification will be included for your protection.

Age…….. Stage of pregnancy……

1. What is your level of formal education? High school / primary school/ university/ no formal education	**2**. Do you live in a House / flat / shack/ homeless
3. How many people share your home?	**4**. (i) How long have you been living at your present address? < 5 Years / 5-10 years / >10 years
5. (i) How many children do you have ? (ii) Are they healthy? Yes / No (iii) If No, what is the problem? (iv) How long has it been?	**6**. (i) Is this your first pregnancy ? Yes / No (ii) If No, how many have you had and did you carry full term? Yes / No (iii) Have you ever had an induced abortion? Yes / No (iv) What stage of your pregnancy was it terminated? First / second / third trimester
7. (i) Have any of your children been born preterm or with low birthweight ? Preterm / low birthweight	**8.** How often do you visit your doctor for gynecological check ups? Never / once a year / only when necessary
9. (i) Do you have frequent urinary tract infections ? Yes / No	**10**. (i) Have you ever had a sexually transmitted disease ? Yes / No (ii) If yes, do you know what it was and was it treated? (iii) How many sexual partners have you had? 1/ <5 /5-10/ ›10
11. (i) How frequently do you visit the dentist ? Never / Once a year / Twice a year / whenever (ii) When was the last time you visited a dentist? (iii) What was the reason? (iv) Do your gums bleed when you brush your teeth? Yes / No (v) How frequently do you brush your teeth? Once a day / twice a day / after every meal (Vi) Do you have bad breath? Yes/ No	**12.** Do you have easy access to medical or dental care? Yes / No
13. Do you have medical insurance? Yes / No	**14.** (i) How often do you have a drink containng alcohol? Never / daily / weekly/ special occasions (ii) How many drinks would you consume when you do drink? 1-2 / 3-5 / >6
15. (i)Do you smoke? Yes / No/ Sometimes (ii)If yes, How many a day? <5 / 5-10 / >10 (iii)How long have you been smoking? <5 / 5-10 />10yrs	**16.** (i) What does your diet mainly consist of ? Bread / meat / fruit and veg /

Thank you for your participation

APPENDIX 2D

Clinical measurements

Patient number: …… **Date of Birth**: ……

Date of examination: …… **Stage of pregnancy**: …….

	16 m/d	21 m/d	24 m/d	36 m/d	41 m/d	44 m/d
PI						
GI						
PD						
LA						
BANA						

Decayed	Missing	Filled	DMF

Observations and comments:

……

……

……

……

……..

Examiner: …… **Assisted by**……

APPENDIX 2E

BANA Solution (Loesche et al. 1987)

Stock solution: 44 mg in 1 ml dimethyl sulphoxide

Working solution: Dilute stock solution 1:100 with Soresen buffer (0.67 mM).

Sorensen's buffer

0.15M KH_2PO_4

0.15M Na_2HPO_4

pH 7.2

at 4^oC

ZYM A

25 g Tris-hydroxymethyl-amino-methane

11 ml HCl (37%)

10 g Lauryl Sulfate

up to 100 ml distilled water

Store at RT. Forms a precipitate at $2\text{-}8^0C$ which is readily dissolved at 68^oC

ZYM B

0.35 % Fast blue BB in 2-methoxyethanol

This reagent is light sensitive and should be kept in the dark, preferably with the container wrapped in foil and stored at 4^oC.

TES buffer

50 mM Tris hydroxymethyl methylamine

pH 8.0

15 mM Ethylenediaminetetra-acetic acid disodium salt.

20 % SDS

20g sodium dodecyl sulfate (SDS) dissolved in 100 ml water. Sterilize by filtration through a 0.2 µm filter.

5 M Ammonium acetate

Dissolve 38.54g ammonium acetate in 100ml distilled water.

10X TE :

100 mM Tris-Cl pH 7.5

10 mM EDT

For 1 liter: Dissolve 12.11 g Tris and 3.72 g EDTA in around 700 ml distilled water. Adjust pH to 7.5 with concentrated HCl. Make volume up to 1 liter. Autoclave.

10XTBE

108g Tris, 55g boric acid and 9.3g EDTA. Dissolve and make up to 1 liter.

Ethidium Bromide (EtBr)

Dissolve 0.1 g in 10 ml of water. Shake well to dissolve. Wear gloves and never breath the dust.

Loading buffer

0.25% bromophenol blue and 0.25% xylene cyanol in 30% of glycerol in distilled water.

APPENDIX 3A

Plaque Indices for all six Ramfjord teeth

	16	21	24	36	41	44
0	59.1%	68.2%	71.2%	62.1%	63.6%	62.1%
1	24.2%	10.6%	19.7%	33.3%	24.2%	31.8%
2	9.1%	3.0%	3.0%	3.0%	10.6%	4.5%
Missing	7.6%	18.2%	6.1%	1.5%	1.5%	1.5%

Gingival Indices for all six Ramfjord teeth

	16	21	24	36	41	44
0	13.6%	36.4%	13.8%	15.2%	28.8%	13.6%
1	57.6%	33.3%	58.5%	62.1%	37.9%	60.6%
2	21.2%	12.1%	21.5%	21.2%	31.8%	21.2%
3	0	0	0	0	0	3.0%
Missing	7.6%	18.2%	6.2%	1.5%	1.5%	1.5%

Pocket Depth for all six Ramfjord teeth

	16	21	24	36	41	44
1	0	1.5%	0	1.5%	4.5%	1.5%
2	9.1%	30.3%	6.1%	12.1%	40.9%	22.7%
3	24.2%	36.4%	39.4%	54.5%	36.4%	48.5%
4	25.8%	6.1%	27.3%	18.2%	13.6%	22.7%
5	22.7%	4.5%	16.7%	10.6%	3.0%	3.0%
6	7.6%	1.5%	1.5%	0	0	0
7	1.5%	0	1.5%	0	0	0
Missing	9.1%	19.7%	7.6%	3%	1.5%	1.5%

Loss of Attachment for all six Ramfjord teeth

	16	21	24	36	41	44
0	68.8%	75.0%	81.3%	89.1%	85.9%	87.3%
1	12.5%	1.6%	9.4%	3.1%	3.1%	4.8%
2	9.4%	1.6%	1.6%	4.7%	6.3%	6.3%
3	0	1.6%	0	0	3.1%	0
Missing	9.4%	20.4%	7.9%	3.2%	1.6%	1.6%

APPENDIX 3B

Prevalence of red complex members in different Ramfjord teeth as determined by PCR

	16	21	24	36	41	44
Missing	7.6%	18.2%	6.1%	1.5%	1.5%	1.5%
Negative	51.5%	57.6%	59.1%	68.2%	57.6%	65.2%
Positive(All 3)	1.5%	-	1.5%	3.0%	3.0%	4.5%
Positive(P.g&T.f)	6.1%	7.6%	9.1%	4.5%	3.0%	4.5%
Positive(P.g)	18.2%	9.1%	13.6%	16.7%	16.7%	9.1%
Positive(T.d&T.f)	4.5%	-	3.0%	1.5%	3.0%	3.0%
Positive(T.d)	3.0%	-	1.5%	1.5%	3.0%	3.0%
Positive(T.d.&P.g)	3.0%	-	3.0%	1.5%	4.5%	1.5%
Positive(T.f)	4.5%	7.6%	3.0%	1.5%	7.6%	7.6%