A COMPARATIVE STUDY OF VIRUSES, BACTERIA & FUNGI PRESENT IN THE PLACENTAL TISSUES OF WOMEN WITH PRE-ECLAMPSIA & NORMOTENSIVE PREGNANT WOMEN USING MOLECULAR GENETIC TECHNIQUES

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DECLARATION

This thesis is my original work and has not been submitted previously for a degree at this or any other University/ Institute to the best of my knowledge. It does not contain any material published or written by any other person, except as acknowledged in the text.

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ABSTRACT

Pre-eclampsia is a hypertensive disorder of pregnancy that affects both the mother and her unborn baby. It is the leading cause of maternal and infant morbidity & mortality. The specific aetiology of pre-eclampsia still remains unclear. Research suggests that the primary pathology of preeclampsia is defective placentation which may be caused by many factors including infectious agents.

Accordingly, the objective of this thesis was to study the viruses, bacteria and fungi present in the placental tissues of women with pre-eclampsia and normotensive pregnant women using molecular genetic techniques. Placental tissue, amniotic fluid, blood and urine samples were collected from 55 primiparous women with pre-eclampsia and 55 primiparous normotensive pregnant women matched for age and body mass index at the time of delivery by caesarean section under stringent aseptic conditions.

The study was carried out in two phases. In phase 1, a 500bp fragment of the 16S and 28S rRNA genes were amplified and Sanger sequenced for the detection of bacteria and fungi respectively. Viral metagenomics was employed for the detection of viruses. In the presence of mixed infections, the amplified 16S and 28S rRNA genes were cloned and sequenced. In addition to the placental tissue samples, amniotic fluid, blood and urine samples were also tested for the presence of bacteria and fungi. In phase 2 of the study the placental tissue samples that were positive for bacteria were further studied using 16S metagenomics technology on an Illumina MiSeq Next Generation platform.

At the end of phase 1, 7(12.7%) placental tissue samples of the 55 women with preeclampsia were positive for the presence of bacteria and identified as *Bacillus cereus* in 1 (14.2%), *Bacillus circulans* in 1 (14.2%), *Stenotrophomonas* in 1 (14.2%), *Pseudoxanthomonas* in 1 (14.2%), *Klebsiella pneumonia* in 2 (28.5%), *Bacillus sp.* in 4 (57.1%), *Lactobacillus iners* in 1 (14.2%) and uncultured bacteria in 3(42.8%). The detection of bacteria in the placental tissue samples were statistically significant (P=0.006). One (1.8%) placental tissue sample obtained from a woman with preeclampsia was positive for the presence of fungi which was identified as *Malassezia restricta*. However none (0%) of the women with preeclampsia were positive for the presence of viruses. Similarly, none (0%) of the placental tissue samples obtained from normotensive pregnant women had bacteria, fungi or viruses.

In addition, 1(2%) of the 48 amniotic fluid samples, 3 (5.5%) of 54 blood samples and 8 (14.5%) of the 55 urine samples obtained from women with preeclampsia were positive for the presence of bacteria. Nine (16%) of the 55 urine samples obtained from normotensive pregnant women had bacteria. All amniotic fluid, blood and urine samples obtained from women with preeclampsia and normotensive pregnant women were negative for the presence of fungi. At the end of phase 2, complete microbiome of the 7 placental tissue samples was obtained.

This study confirms the presence of bacteria in the placental tissues of a subset of women with pre-eclampsia and support the role of bacteria in the multi factorial aetiology of pre-eclampsia.

ACKNOWLEDGEMENT

"Life is like an Ocean, it can be calm or still, rough or rigid, turbulent at times but in the end it is always beautiful."

The unassuming but yet huge service carried out by the medical fraternity, and their sacrifice attracted me to medicine at a very young age. As the years went by and as we reached the gradual ages of maturity, under the guidance of some great teachers and our parents, we saw the world and the reality. It was an age of sincerity guided by principled people. Certain books and documents we were encouraged to read enhanced my interest in medicine and the medical field. Reading on research fascinated me, "prevention over cure" made me to choose a path in the research field.

With the dawn of the New Year in 2009, I had just returned after completing my MSc from the University of Westminster London, a new chapter turned in my life and the opportunity of realizing my dreams in following a field of research came true. Few words may never express our sincere gratitude to Prof. Vajira Dissanayake for the faith he displayed, entrusting me with one of his main and a priority project in identifying a common cause for preeclampsia. It was no easy journey. Professor Vajira guided us and worked side by side with us leading from the front to accomplish our mission. His determination and dedication to serve saw us through many difficult encounters.

Turbulent times were there, times of happiness and disappointment, times of desperation, but Prof. Vajira Dissanayake and Prof. Rohan Jayasekara stood as pillars and there was no return until and otherwise the mission was accomplished, the destination reached. They never let go standing firmly beside you, checking and correcting every faulty step. The facilitation and funding provided by the Human Genetics Unit under the guidance of Prof. Vajira Dissanayake

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I remember, with utmost sincerity the team at Human Genetics Unit, Faculty of Medicine Colombo. My colleagues who stood by me for four and a half years tolerating, encouraging and supporting me right through out. Human Genetics Unit will always be a memorable place for me where I found great friends, personalities always with words of encouragement and wisdom. A special word of thanks to my seniors Dr. Gayani, Dr. Nilakshi, Dr. Kalum & Dr. Chamila for the unwavering support guidance and words of advice.

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We have reached the end of a chapter which would be the beginning of a new one. As Prof Vajira Dissanayake insists the book would be closed only the day prevention or an ultimate cure is identified for pre-eclampsia; the day the expectant mothers and babies would be beyond the reach of such deadly diseases.

DEDICATION

My journey was made possible and made beautiful by two amazing human beings. They motivated us and cultivated the passion in us to achieve. They guided and inspired us. They went through many hardships and faced many challenges, yet instilled confidence in us to dream and achieve our goals. **'Ammi & Appachchi',** thank you for loving me unconditionally. This thesis is dedicated to you both with love and gratitude.

Table of Contents

LIST OF FIGURES

LIST OF TABLES

1 INTRODUCTION

1.1 Definition of 'Pre-eclampsia'

The hypertensive disorders of pregnancy are conditions associated with high blood pressure, proteinuria and in certain cases convulsions during pregnancy. The International Society for the Study of Hypertension in Pregnancy (ISSHP) defined pre-eclampsia for research purposes as *de novo* occurrence of hypertension (systolic blood pressure (SBP) ≥140 mmHg and diastolic blood pressure (DBP) ≥90 mmHg) on two occasions 6 hours apart occurring after 20 weeks of gestation in a previously normotensive pregnant woman, which returns to normal by the end of the third month postpartum, in association with proteinuria (≥300 mg in a 24 hour collection or ≥500mg/L on a spot urine collection or ≥30 mg protein/mmol creatinine in a spot urine collection or, failing other measurement, ≥1+ on dipstick testing) (Brown, 2001, Dissanayake et al., 2004) not associated with urinary tract infection or ruptured membranes.

The presence of oedema, which was used in the past as a necessary feature for the diagnosis of pre-eclampsia is no longer being used and has now been removed from both clinical and research definitions as it is not specific to the condition.

1.2 Importance of pre-eclampsia

1.2.1 Morbidity, mortality and incidence of pre-eclampsia in Sri Lanka

Sri Lanka is a small country with a land area of 62700 km^2 and a population of approximately 20 million. The population of females in Colombo the capital of Sri Lanka, is estimated to be around 1.2 million and female literacy is around 89.7% (Statistics, 2005). The gross national income per capita approximates to US$ 1540. Whilst 1.8% of the gross domestic product is spent on health care, the spending on maternal and child health remains at 0.23% (Senanayake et al., 2011).

During the year 2010, Colombo has recorded 56,326 live births with a population growth rate of 1.4 (UNFPA, 2007). Pregnancy or the expectant arrival of a new baby is an important phase of life, not only for the mother to be, but also for the whole family, and will remain a unique event in any culture. The mother to be and families rejoice with the greatest gift mankind would ever receive. To have hopes dashed and lost forever due to the loss of a life of the mother or child and in certain instances of both as a result of complications that arise during the pregnancy period remains a massive concern for the entire world.

For decades, hypertension during pregnancy has been one of the leading causes for maternal deaths in Sri Lanka (Prathapan, 2012). Pre-eclampsia remains a vital and a more complicated disorder not only due to the deaths associated with the condition but the permanent damage it could cause to certain crucial organs of the mother.

Pre-eclampsia is not a stranger or a recent discovery in Sri Lanka. According to Senevirathne, 2009, the first publication on eclampsia in Sri Lanka appeared in the Ceylon Medical Journal in the year 1891 in which Attygalle and Neli provided a classical description on pre-eclampsia/ eclampsia, the natural progression related to the disease and management. Maternal deaths due to hypertension during the period 1931 to 1935 ranged between 57 to 81.45% which has gradually decreased to 11.8% by 1940 due to improvements in antenatal care (Senevirathne, 2009).

The maternal mortality rate of Sri Lanka (Figure 1.1) stood at 33.4 per 100,000 live births in 2008 (Senanayake et al., 2011) and infant mortality rate stood at 12 per 1000 live births during the year 2003 (Statistics, 2005).

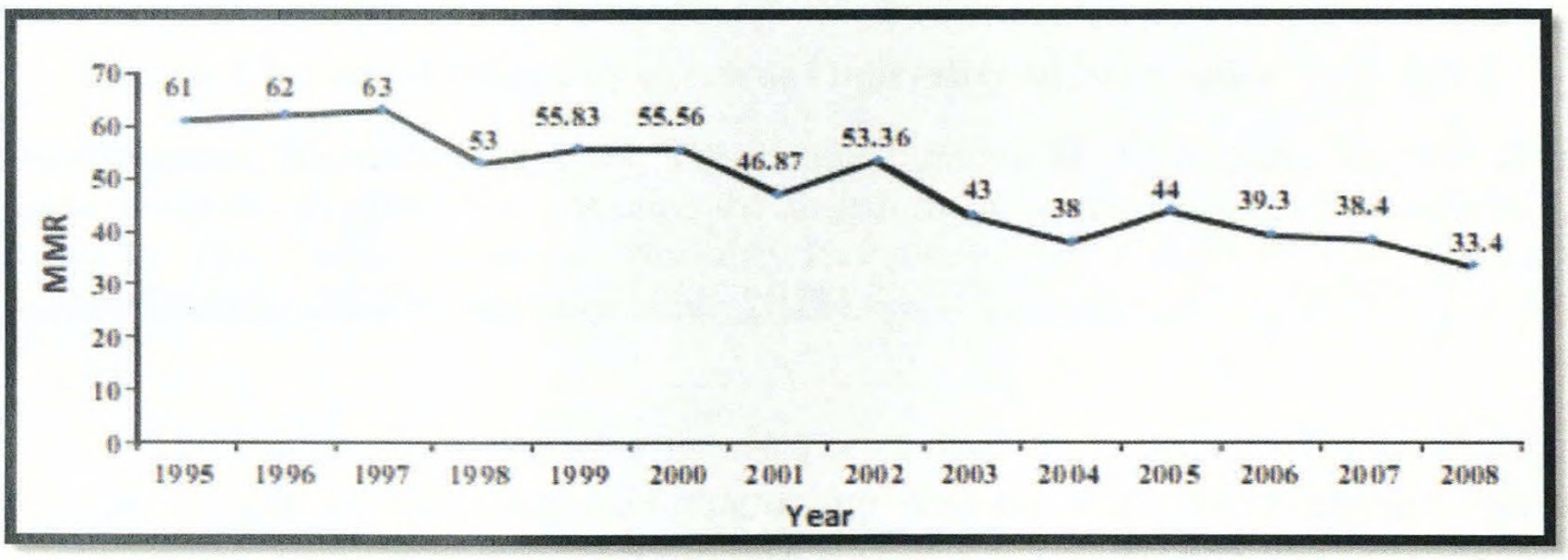


Figure 1.1 Maternal mortality ratio of Sri Lanka 1995-2008

The maternal mortality rate of Sri Lanka was high at 61 per 100,000 live births in 1995 and has decreased to 33.4 per 100,000 live births in 2008. Annual Maternal Mortality Review, Family Health Bureau, Colombo, Sri Lanka (Senanayake et al., 2011)

Hypertensive disorders accounted for 10.3% of maternal deaths in 1997 (Family Health FHB, 1998). In 2007 maternal deaths were reported as 7% (Figure 1.2) against neonatal deaths of 24% (Senanayake et al., 2011).

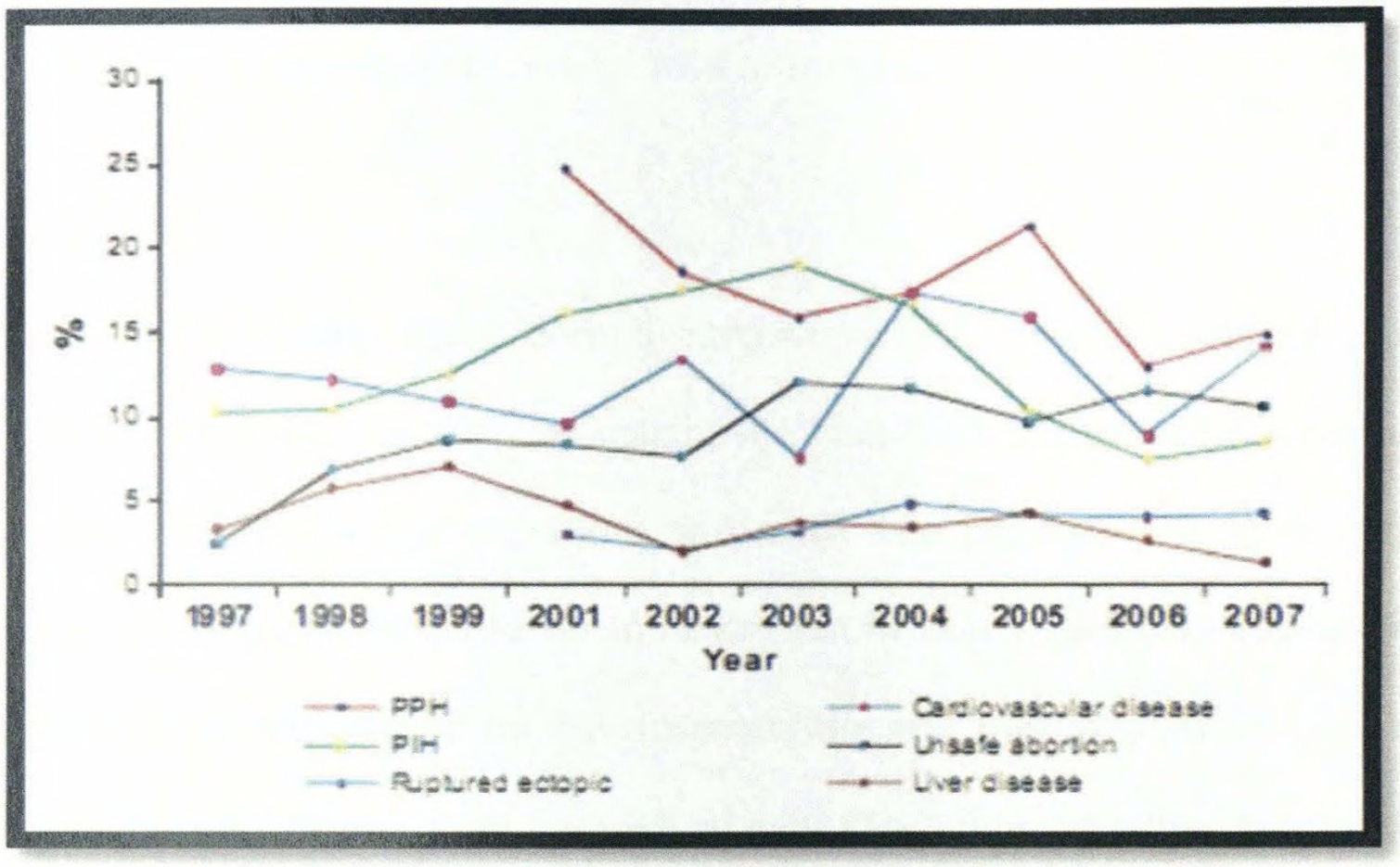


Figure 1.2 Causal trends of maternal mortality in Sri Lanka 1997-2007

PPH, Post-Partum Haemorrhage; PIH, Pregnancy Induced Hypertension. In year 2007 the Pregnancy Induced Hypertension remains the fourth most common cause of maternal deaths accounting for 7%. Annual Maternal Mortality Review, Family Health Bureau, Colombo, Sri Lanka, unpublished data (Senanayake et al., 2011)

A study carried out in 2007 reported that many women with pre-eclampsia had severe pregnancy complications where, 75.5% of women had systolic blood pressure of ≥ 160mmHg, 83.8% of women had diastolic blood pressure of ≥ 110mmHg and 87% of women suffered of proteinuria ≥ 3+. The report also indicated that 38.3% of women delivered at <34 weeks and 48% of babies born to women with pre-eclampsia were small for gestational age. The study which had been carried out at two tertiary care hospitals in Sri Lanka had an incident of one maternal death and 25% of high prenatal mortality rate (Dissanayake et al., 2007).

It is observed that Sri Lanka has progressed satisfactorily in maternal health care over the years. It is believed that free health care services and free education facilities available in Sri Lanka has contributed immensely towards such achievements. However a substantial amount of pregnancy related complications associated with pre-eclampsia remains yet to be addressed and needs to be addressed soon (Dissanayake, 2004, Zhang, 2003). Pre-eclampsia in the meantime continues to contribute to nearly 50% of maternal deaths (Attygalle, 2011).

Early onset of pre-eclampsia under normal circumstances is related to an enhanced risk of potentially fatal complications such as eclampsia, haemolysis, elevated liver enzymes and placental abruption (van Rijn et al., 2006) and as of date pregnancy induced hypertension remains the fourth commonest cause for maternal deaths in Sri Lanka as indicated in Figure 1.2. Pre-eclampsia is responsible for severe morbidity and mortality caused by 'Iatrogenic Prematurity'. The only known 'cure' for such adverse conditions remains delivery of the baby or the placenta. Many babies born to mothers whose pregnancies are complicated with pre-eclampsia undergo pre-term delivery with severe neonatal morbidity and mortality risks (Senanayake et al., 2011).

This situation highlights the need for special care and attention for the mothers and babies in pregnancies complicated with pre-eclampsia. In a study carried out in Sri Lanka, 34% of infants reported to have been born to women with pre-eclampsia required special care (Jayawardana, 1994). The Perinatal Mortality Rate (PMR) in Sri Lanka accounted for 25% in 2004 (Dissanayake, 2004) and 24% in 2011 (Senanayake et al., 2011). This is comparatively high to the PMR of the west which remains at 5% (Zhang, 2003). Under these circumstances, the necessity to further reduce the maternal and neonatal morbidity and mortality in Sri Lanka

remains a priority. It is a fact that Sri Lanka is yet a long way away in reaching its goal towards achieving a single digit maternal mortality rate providing safe motherhood to all women (Prathapan, 2012).

1.2.2 Morbidity, mortality and incidence of pre-eclampsia globally

"Life is precious in all context and no woman should die giving life" (UNFPA, 2007). Safe motherhood is a right of every woman and it is unacceptable when pregnancy kills. It is indeed distressing to note that everyday around 800 women worldwide die from pregnancy and child birth related complications (WHO, 2012). Every year, 10-15 million women suffer from the aftermaths of pregnancy related complications and pre-eclampsia remains one of the key causes of maternal death (UNFPA, 2007). It is estimated that pre-eclampsia is responsible for 50,000 maternal deaths (WHO, 2011) and 5-7% of all pregnancy complications worldwide annually (Sarsam et al., 2008, Wagner, 2004, Thangaratinam et al., 2008, Gaillard et al., 2011b, Ghulmiyyah and Sibai, 2012). Though many studies exist on the incidence of pre-eclampsia, assessing the epidemiology of the disease or the incidence of hypertensive disorders of pregnancy remains somewhat difficult due to the inadequate standards followed (Dolea, 2003).

However the impact of the disease is felt more severely in developing countries in comparison to the developed countries (Adamu et al., 2003, Osungbade and Ige, 2011, Igberase and Ebeigbe, 2006, Irgens et al., 2001). A woman is 7% more likely to develop pre-eclampsia in developing countries compared to the developed countries and ten million women develop pre-eclampsia each year around the world (Preeclampsia_Foundation, 2013). The risk of severe morbidity associated with hypertension in pregnancy is estimated to be ten times higher than the maternal mortality rate (Kuklina et al., 2009).

In the United States pre-eclampsia is responsible for around 6-10% of pregnancy complications and accounts for 15.9% of maternal deaths (Backes et al., 2011). Also, the number of women hospitalized due to pregnancy induced hypertension including pre-eclampsia is on the increase in the United States day by day and the majority of the cases are associated with severe obstetrics morbidity. A study carried out during the period 1998- 2006 reveals that women hospitalized for hypertension had a higher risk of adverse pregnancy complications compared to the normotensive pregnant women being hospitalized (Kuklina et al., 2009). In African, Latin American and the Caribbean countries it was responsible for 20-25% maternal deaths (Duley, 1992). Recent reports indicate an incidence rate of 3.3% in Australia (Thornton et al., 2013). Nigeria has a prevalence rate between 2 to 16.7% with pre-eclampsia being the major cause of maternal deaths (Osungbade and Ige, 2011). In Nigeria the risk of maternal death due to pregnancy complications is about 1 in 7 whilst in Ireland it remains at 1 in 48,000 (UNFPA, 2010).

In South Africa, pre-eclampsia is the second major cause of maternal deaths and in United Kingdom it is believed to be the fourth most common cause (Dyer et al., 2007). African countries such as South Africa, Egypt, Tanzania, and Ethiopia record a range between 1.8 to 7.1% (Mahaba et al., 2001, Osungbade and Ige, 2011, Kimbally et al., 2007, Thiam et al., 2003, Teklu and Gaym, 2006). The incidence of eclampsia in the developed countries of North America and Europe is estimated to be around 5 to 7 cases per 10,000 deliveries whereas 1 case per 100 pregnancies is recorded in developing countries (WHO, 2004). Globally, 10-15% of direct maternal deaths are associated with pre-eclampsia and eclampsia (Duley, 2009) and there is a fivefold increase in perinatal mortality following pre-eclampsia (Brown et al., 2001). Accordingly severe maternal morbidity associated with preeclampsia is not only restricted to

Sri Lanka but remains a global problem which awaits to be addressed as a priority (Zhang, 2003).

Pre-eclampsia leads to complications in the liver, kidney, brain and clotting system. In addition, maternal complications have a negative effect on neonatal health with an increasing risk of prematurity, poor growth and fetal death. Over 3 million neonatal deaths are reported each year due to obstetrics complications (Cousens et al., 2011). 12% of babies born to women with pre-eclampsia are smaller for gestational age (SGA) and 1/5th of them are premature. In the United Kingdom, 6% of babies born to women with pre-eclampsia die annually. In developing countries around, 27% of neonatal deaths are caused by pre-eclampsia and eclampsia (Duley, 2009). As discussed, the incidence of pre-eclampsia varies from country to country. It is difficult to deduce accurately the incidence of pre-eclampsia. This variation may be due to variations in diagnosis and clinical management of the condition. It is evident that epidemiological studies carried out over the years indicates no reduction in the incidence of pre-eclampsia associated with the overall burden of life threatening severe obstetrics complications. There are no reliable markers for prediction and detection of pre-eclampsia and early delivery places the life of the foetus at a massive risk (Kuklina et al., 2009).

1.3 Patho-physiology of pre-eclampsia

Preeclampsia is a disease limited to human pregnancies and its aetiology still remains a mystery. Pre-eclampsia is now acknowledged to be a two-stage disorder (Dissanayake, 2004, Roberts, 2000, Redman and Sargent, 2005).

Stage 1- Defective placentation

Stage 2- Maternal syndrome characterized by endothelial dysfunction

1.3.1 Defective placentation

The only assured cure so far known for pre-eclampsia is termination of the pregnancy by the delivery of the placenta and the foetus. This would resolve the adverse maternal condition which persisted within days if not hours. However, it is also evident from observations made over the years that 'cure' depends on the delivery of the placenta rather than foetus. Accordingly, it is clear that pathophysiology of the placenta is primarily responsible for the disorder rather than fetal elements of pregnancy (Jeffcoate, 1966). The placenta is well known to play the key role in the development of pre-eclampsia thus making it a disorder of pregnancy (Carty, 2011).

During normal pregnancy, cytotrophoblast cells deriving from the anchoring villi of the fetal part of the placenta invade the maternal endometrium. This process is known as interstitial invasion. A portion of these extra villous trophoblast cells attain endothelial characteristics and invades maternal spiral arteries which process is known as endovascular invasion. During early pregnancy, these trophoblast cells help in maintaining a hypoxic uterine environment (Pennington et al., 2012). They eventually substitute some of the endothelial cells in the vessel

wall and make the vessel wall 'leaky', filling inter villous spaces of the placenta with maternal blood as shown in Figure 1.3 (Kaufmann et al., 2003, Hunkapiller and Fisher, 2008, Pennington et al., 2012).

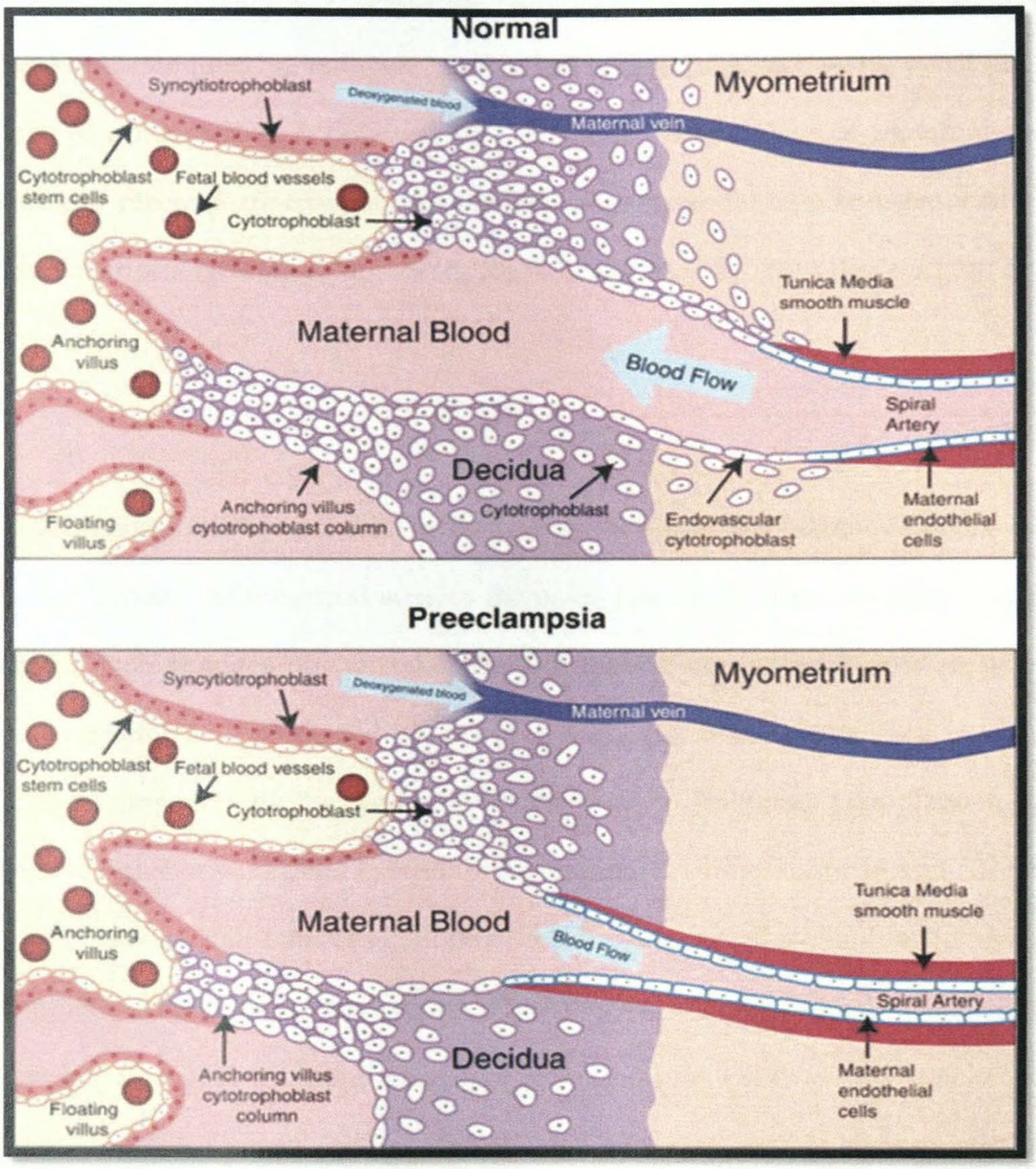


Figure 1.3 Abnormal placentation in women with pre-eclampsia

Women with preeclampsia have shallower invasion of spiral arteries and reduced trophoblast invasion, resulting in an abnormal placentation (Lam et al., 2005)

As illustrated in figure 1.3, invasion of the spiral arteries in women with preeclampsia is shallower and these arteries remain as small calibre resistant vessels with reduced placental perfusion (Lam et al., 2005) due to the smaller number of trophoblast cells present within the spiral arterioles, which makes the vessel wall rigid (Khong et al., 1986, Aquilina and Harrington, 1996). Consequently, reduced trophoblast invasion is an early event in the development of pre-eclampsia. It is believed that abnormal remodelling of maternal spiral arteries deprives the placenta of oxygen and that the consequential hypoxia generates the symptoms of preeclampsia (Khong et al., 1986, Roberts and Hubel, 2009, Pennington et al., 2012).

During normal pregnancy, trophoblastic invasion is associated with prominent changes in the spiral arteries. The diameter of the spiral arteries increases four to six times. However, unlike normal pregnancy, such changes do not take place or remain considerably low in women suffering from pre-eclampsia. A 40% reduction in the spiral arteries was observed in women with pre-eclampsia compared with the normal pregnant women. They may take place in other external decidual vessels but would not extend to the inner third of the myometrium (Roberts, 2000, Roberts and Catov, 2008, Lam et al., 2005). This leads to failed trophoblastic invasion leading to incomplete remodelling of the uterine spiral arteries, causing defective placentation resulting in unfavourable obstetric conditions like pre-eclampsia (Enders and Blankenship, 1997, Harendra et al., 2011, Gomez and Parry, 2009, de Groot et al., 2005, Hung et al., 2002, Gilbert et al., 2008). Research findings also suggest that infection specific inflammation plays a role in trophoblast complications which leads to defective placentation causing pre-eclampsia. It is reported that pre-eclampsia patients with an infection had an altered balance between proliferation and apoptosis of villous trophoblast cells in the placental tissues (Padmini, 2011).

It is evident from past studies that babies born to women with early onset pre-eclampsia were born pre-term and were small for gestational age (SGA) or were experiencing intra uterine growth reduction (IUGR) compared to the babies born to women with late onset pre-eclampsia. Abnormal placentation leading to reduced placental perfusion will limit the nutrition and oxygen transported across and will result in IUGR and SGA babies (Roberts and Catov, 2008). Even though the pathophysiology of defective placentation is known, the aetiology contributing to the defective placentation still remains unknown.

1.3.2 Maternal syndrome

The second stage is the maternal response to defective placentation characterized by the primary clinical picture of hypertension and proteinuria (Lam et al., 2005). As shown in to figure 1.4, reduced placental perfusion secondary to defective placentation may result in a state known as 'ischaemic placenta'. This releases a range of factors into the maternal circulation which causes endothelial dysfunction leading to the development of pre-eclampsia (Pennington et al., 2012, Carty, 2011, Lee et al., 2007, Redman and Sargent, 2010).

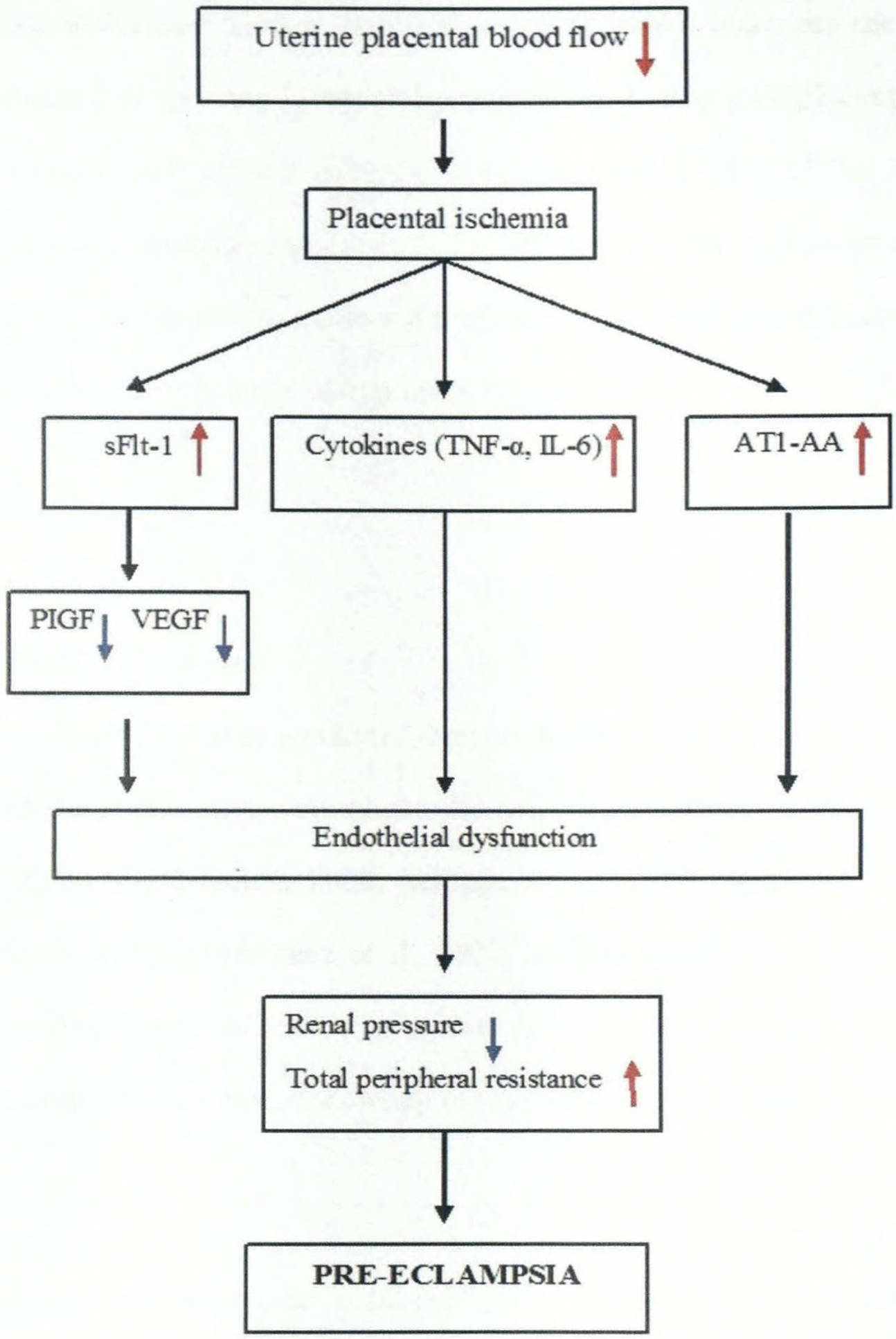


Figure 1.4 Placental ischemia and hypertension

The decreased uterine blood flow causes placental ischemia which leads to endothelial dysfunction during pregnancy leading to preeclampsia. sFlt1, soluble forms like tyrosine kinase 1; TNF- α, Tumor Necrosis Factor; IL-6, Interleukin 6; AT1-AA, angiotensin receptor auto antibodies ; PIGF, Placental Growth Factors, VEGF, Vascular Endothelial Growth Factor

The decreased uterine blood flow causes placental ischemia which increases the levels of cytokines, soluble forms like tyrosine kinase-1 leading to the decrease of placental growth factors and vascular endothelial growth factors causing endothelial dysfunction. This utero placental perfusion pressure directly contributes to the arterial pressure and proteinuria. This in turn results in altered renal function, increased total peripheral resistance and intra uterine growth restriction which ultimately leads to hypertension causing pre-eclampsia (Gilbert et al., 2008).

1.3.3 Link between the two phases

The placental initiated 'toxins' such as cytokines, anti-angiogenic factors, syncytiotrophoblast microparticles (STNM) and oxidative stress links the two stages; defective placentation and maternal response (Roberts and Hubel, 2009, Schipper et al., 2005, Maynard et al., 2005, Redman and Sargent, 2005, Mellembakken et al., 2002, Roberts and Hubel, 1999). Although such factors are most frequently considered as toxins, this could be a signal from the fetal-placental unit to overcome the nutrition deficiency in the placenta (Roberts and Hubel, 2009).

Maternal manifestation of pre-eclampsia is thought to be mediated by soluble forms like tyrosine kinase-1 (sFlt-1) and soluble endoglin (sEng) (Figure 1.4) which are the soluble anti angiogenic factors synthesized by the placenta. Lam, C. et al suggests that high blood pressure and proteinuria characterizing pre-eclampsia is due to excess circulating soluble forms like tyrosine kinase. This sFlt-1 is an endogenous antiangiogenic protein synthesized by the placenta and is involved in deactivating and obstructing the signalling action of vascular endothelial growth factor (VEGF), placental growth factor (PLGF) and transforming growth factor β (TGF), there by significantly increasing the ratio of sFlt-1 to PLGF resulting in poor

placental development (Lam et al., 2005, Levine et al., 2004). Loss of signals to the glomerular endothelium results in the breaking down of the renal filtration barrier causing proteinuria which finally leads to placental ischemia (Parikh and Karumanchi, 2008, Maynard et al., 2008, Venkatesha et al., 2006, Levine et al., 2006, Levine et al., 2004).

Defective placentation may increase the soluble antiangiogenic factors in the systemic circulation which in turn would injure the maternal blood vessels leading to hypertension, proteinuria and finally causing damage to the organs throughout the body (Parikh and Karumanchi, 2008, Zhou et al., 2008, Wu et al., 2010).

Whilst surveying the literature, an interesting aspect observed was that the previous studies too provide evidence of the presence of infectious agents as a factor linking the two stages of pre-eclampsia. It is suspected that an infectious agent or agents may be responsible for the activated blood components, excess sycytiotrophoblast (STBM) fragments, activating immune cells, transforming oxidized lipids or excess inflammatory cytokines (Roberts and Hubel, 2009). Placental oxygen deregulation (oxidative stress as an imbalance of pro and anti-oxidative factors), abnormal trophoblast invasion, inappropriate maternal vascular damage and anomalous maternal-fetal immune interactions are expected to cause endothelial dysfunction (Pennington et al., 2012).

1.4 Aetiology contributing to defective placentation

Even though the pathophysiology of pre-eclampsia is known, the aetiology contributing to the defective placentation remains unknown. Genetics, environmental factors, social factors, and association with infections are believed to influence defective placentation and maternal response (Rasmussen and Irgens, 2008, Rasmussen et al., 2000, Roberts and Catov, 2008, Parikh and Karumanchi, 2008).

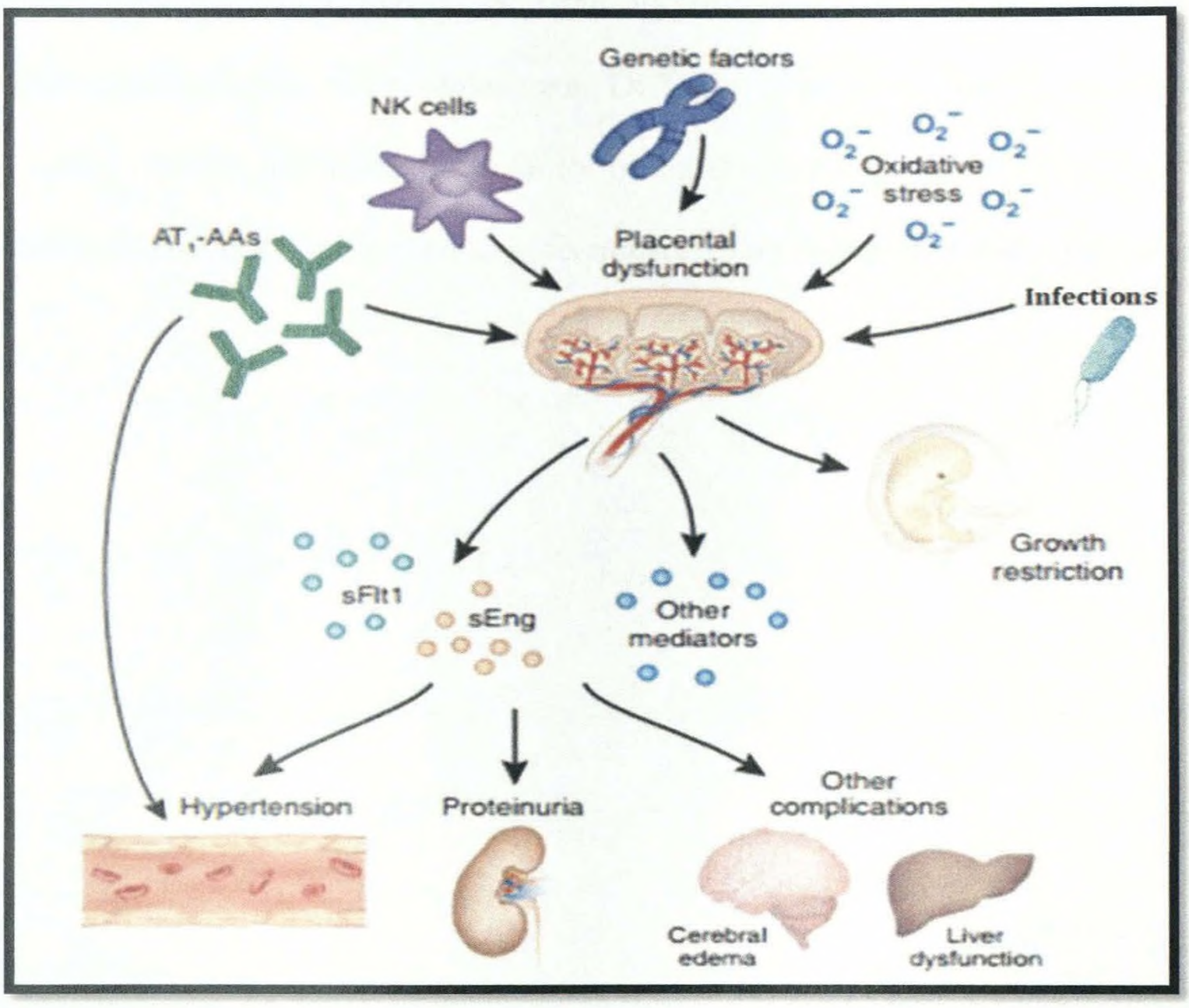


Figure 1.5 Aetiology contributing to defective placentation

Genetic factors, immunological factors, angiotensin receptor autoantibodies and infections may cause placental dysfunction leading to hypertension, proteinuria and growth restrictions (Parikh and Karumanchi, 2008)

As shown in Figure 1.5, it is believed that angiotensin receptor autoantibodies (AT_1-AA_s), oxidative stress, genetic factors or an infectious agent (infection) may cause placental dysfunction leading to the release of antiangiogenic factors (sFlt-1 and sEng) and other inflammatory mediators developing pre-eclampsia (Parikh and Karumanchi, 2008, Carty, 2011).

As indicated in Figure 1.6, the causes of abnormal placentation and causes for the increasing of anti-angiogenic factor still remains uncertain. Finding answers to these questions would be the key to solving the mystery of pre-eclampsia. Different hypotheses put forward by scientists' states that genetic factor, immunological factor or some other factor could be the origin of the disease which may cause either abnormal placentation or an increase in anti-angiogenic factors.

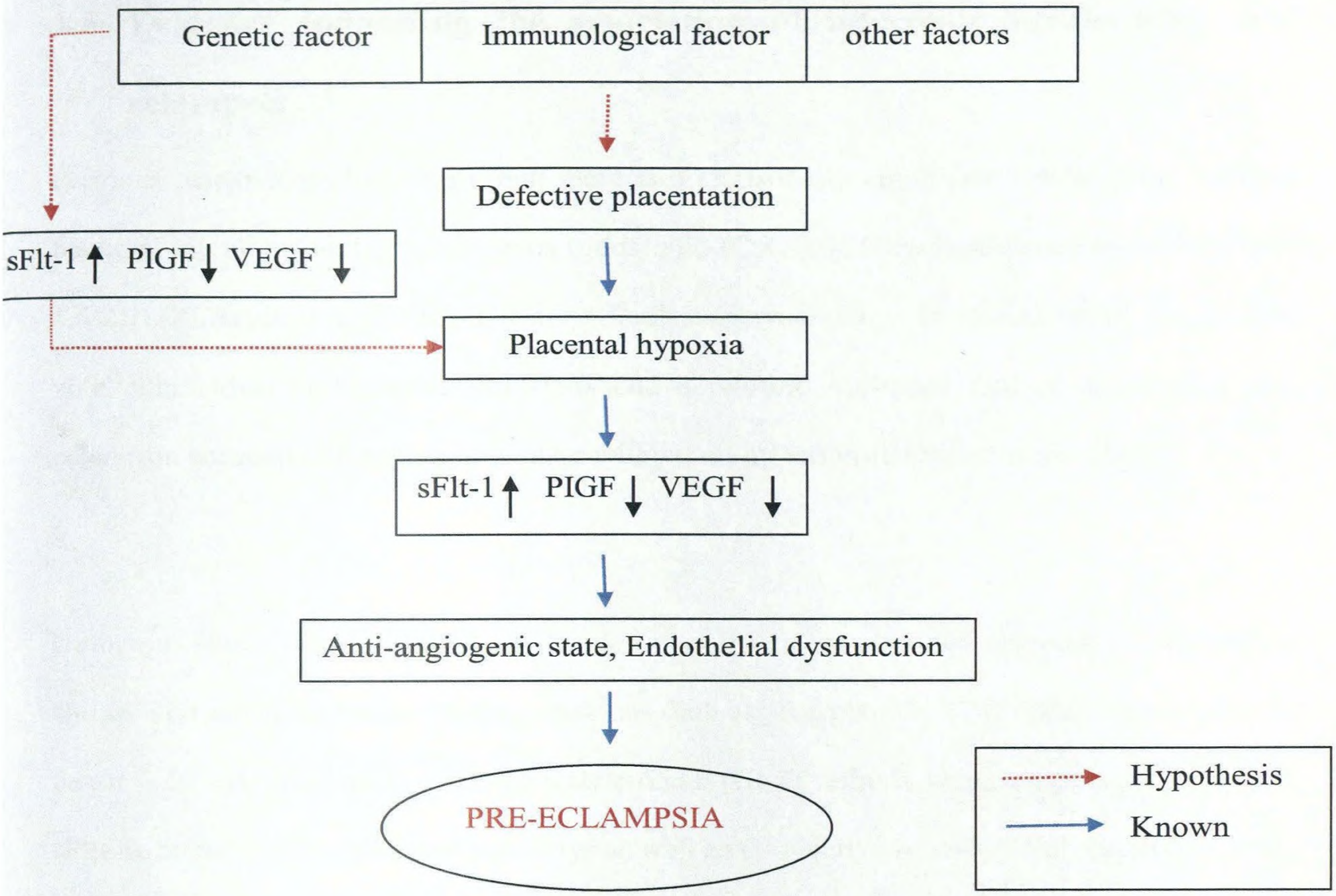


Figure 1.6 Aetiology contributing to the pathogenesis of pre-eclampsia

Defective placentation causes endothelial dysfunction leading to preeclampsia. Genetic factors, immunological factors may cause defective placentation or anti angiogenic state leading to placental hypoxia. sFlt1, soluble Forms like tyrosine kinase 1; PIGF, Placental Growth Factors, VEGF, Vascular Endothelial Growth Factor (Bdolah et al., 2005)

1.5 Evidence suggesting the association of infectious agents with pre-eclampsia

Previous research studies imply that there is a statistically significant relationship between maternal infections and pre-eclampsia (odds ratio (OR) 2.1; 95% Confidence interval (CI) of 1.6-2.7) (Rustveld et al., 2008). The meta- analysis carried out on 16 studies report that women with either viral or bacterial infections had a twofold increased risk of developing pre-eclampsia compared to pregnant women without an infection (Rustveld et al., 2008).

Numerous studies demonstrated an elevated immune response in women with pre-eclampsia and an increase in the inflammatory cytokines such as prostglandin E2 (PGE2), tumor necrosis factor (TNF-α), interleukin 1 (IL-1), interleukin 6 (IL-6), soluble phospholipids A2, activated clotting factor and complement pathways as well as C- reactive protein (CRP) (Rustveld et al., 2008, Sacks et al., 1998, Rinehart et al., 1999, Visser and Wallenburg, 1999, Sanchez et al., 2000, Amory et al., 2001, Benyo et al., 2001, Luppi et al., 2002, Paternoster et al., 2006, Eiland et al., 2012, Djurovic et al., 2002, Kronborg et al., 2011, Lynch et al., 2010). Besides, other inflammatory response markers such as calprotectin, neopterine and some acute phase proteins such as ferritin, C- reactive protein, fibrinogen also would increase due to infections occurring before or during pregnancy elevating the risk of pre-eclampsia (Holthe et al., 2005, Kaleli et al., 2005, Lopez-Jaramillo et al., 2001). In line with the above observation, some studies have shown a profusion of soluble markers of neutrophil activation in pre-eclampsia suggesting the role of infection in association with pre-eclampsia (Eiland et al., 2012, Shamshirsaz et al., 2012, Szarka et al., 2010, Sharma et al., 2007, Teran et al., 2001). In addition, remarkable activation of blood leukocytes (granulocytes and monocytes) were observed in women with pre-eclampsia compared to healthy pregnant women (Redman and Sargent, 2003).

Long-range uteroplacental hypoxemia may induce inflammatory changes in the placenta with the release of inflammatory chemokines, stimulates fetal neutrophils and monocytes, which in turn, may further release inflammatory mediators. This would create a vicious circle, leading to increased endothelial cell activation (Mellembakken et al., 2001). This was observed in a study carried on venous cord blood collected from 36 neonates born after uncomplicated pregnancy and 35 neonates born pre-term to women with severe pre-eclampsia. Neonates born to women with pre-eclampsia had an increased expression of CD15s (P50.003), CD49d/CD29 (P50.01/0.005), and CD31 (P50.007) on neutrophils and CD15s (P, 0.001), CD11c (P50.009) and CD54 (P50.001) on monocytes (Mellembakken et al., 2001).

Subsequently, it was noticed that the systemic inflammatory response turns out to be radically high in women with pre-eclampsia compared to normotensive women and that led to the belief that infection might be associated with the aetiology of pre-eclampsia by increasing the uteroplacental atherosis and by amplifying the maternal systemic inflammatory response (Villar, 2008, Redman and Sargent, 2005, Herrera et al., 2001, von Dadelszen and Magee, 2002).

Research carried out by Von Dadelszen demonstrated that women with early onset pre-eclampsia had elevated amounts of IgG to *C. pneumoniae* (von Dadelszen and Magee, 2002). The results were replicated in another study where, serum samples tested on 37 women with pre-eclampsia and 37 normotensive women indicated that women with high titres of IgG to *C. pneumoniae* had three times the risk of developing pre-eclampsia compared to the normotensive group (Heine, 2003).

Although a similar study carried out by Goulis et al was not able to relate an association of *C. pneumoniae* with pre-eclampsia, antibodies detected against *C. pneumoniae* were significantly higher ($p \leq 0.003$) in women with pre-eclampsia compared to normotensive women (Goulis et al., 2005). With the evidence of past research it is suggested that pre-eclampsia is associated with a chronic infection rather than an acute infection or re-infection (Heine, 2003, Goulis et al., 2005).

Xie, F. reported that infection with *C. pneumoniae* and Cytomegalo virus would trigger the maternal inflammation response and consequently would lead to the development of pre-eclampsia (Xie, 2010). *C. pneumoniae* is considered a bacterial agent responsible for human respiratory tract infection that contributes to the pathogenesis of atherosclerosis (Xie, 2010). Literature available on the subject also supports the view that pre-eclampsia and atherosclerosis share a common pathophysiology and are associated with similar risk factors (Roberts, 2000). In addition, women with a history of pre-eclampsia have an increased tendency to develop atherosclerosis in the future compared with normotensive women (Haukkamaa et al., 2009, Aral et al., 2006).

In another study it was reported that *Chlamydia pneumoniae* infection can reduce trophoblast invasion of the uterine wall and is associated with pre-eclampsia (Gomez and Parry, 2009). It was also reported that trophoblast cell viability and invasion were decreased after an infection ($P<0.05$) in women with pre-eclampsia (Gomez and Parry, 2009). Thus the above studies imply that there is a considerable association of infectious agents with pre-eclampsia which brings out the need for further research.

Adding to these findings, a study carried out by Todros et al using a cohort of 417 pregnant women treated with spiramycin against *Toxoplasma gondii* and 353 low risk women who did not take any antibiotic during pregnancy, indicated that women treated with spiramycin during pregnancy had a lower risk of developing pregnancy induced hypertension and preeclampsia possibly by preventing the occurrence of an infection that could complicate the pregnancy. The odds of developing the disease were significantly lower in the treated women (OR= 0.092, 95% CI 0.021, 0.399; $P < 0.001$) (Todros et al., 2006). Further to this, antibiotic prophylaxis was also found to reduce the risk of pre-eclampsia in primigravidae women with a history of urinary tract infection (Mittendorf et al., 1996, von Dadelszen and Magee, 2002).

In a retrospective study carried out on 13852 pregnant women, women with urinary tract infection had a fourfold risk of developing pre-eclampsia compared to the normal pregnant women (($p<0.001$)(OR 4.2,95% CI 1.1-5.1)) (Hsu and Witter, 1995). In another two prospective studies, women with pre-eclampsia were detected positive for the presence of *Ureaplasma urealyticum* and *Gardnerella vaginalis*. It is interesting to note that women who were diagnosed positive for bacteriuria had a 3 fold increased risk of developing pre-eclampsia ($p=0.02$) (Gilbert et al., 1986). A similar study carried out by Hill in the same year reported that asymptomatic bacteria (>100,000 colonies/ml) were more prevalent in women with pre-eclampsia. Out of 100 women with pre-eclampsia recruited to the study, 19% had bacteriuria (Hill et al., 1986). Further, another study carried out by Mittendorf on 386 cases of women with pre-eclampsia and 2355 controls of normotensive women indicated a fivefold risk of developing pre-eclampsia when women were suffering from urinary tract infection (Mittendorf et al., 1996).

Further studies provide evidence of the association of periodontal diseases with pre-eclampsia. In a study carried out by Canakei et al, of a population of 41 cases and 41 matched controls 46.3% of women with pre-eclampsia were positive for periodontal bacteria compared to the 21.9% in the control group. Risk for pre-eclampsia associated with periodontitis was 3 fold (OR 3.5; 95% CI 1.1-9.3) (Canakei, 2004). Similar to this study another study carried out on a larger sample of 763 cases and controls confirmed that periodontal disease increased the risk of pre-eclampsia by two folds (OR 2.4; 95% CI 1.1-5.3) (Boggess et al., 2003). Accordingly, it is assumed that periodontal infection is associated with endothelial damage which results in pre-eclampsia (Shub et al., 2006, Xiong et al., 2006).

There is increasing evidence to suggest that periodontal disease is associated with severe maternal complications which may result due to poor maternal oral health (Parahitiyawa et al., 2009, Lopez et al., 2005, Lopez et al., 2002, Klebanoff and Searle, 2006, Tarannum and Faizuddin, 2007). This could be due to the up regulation of levels of cytokines and other inflammation modulators as a result of infection (Parahitiyawa et al., 2009). Similar results were revealed by Contreras et al, where chronic periodontitis were diagnosed among 63.8% pre-eclamptic women compared to the 36.6% of normotensive women. In addition, *Porphyromonas gingivalis* (OR 1.8; 95%CI1.1-2.8), *Tannerella forsythia* (OR 1.8; 95% CI1.1-3.0), and *Eikenella corrodens* (OR 1.8; 95%CI1.1-2.8) were also detected from samples obtained from women with pre-eclampsia (Contreras, 2006). This further supports the association of infectious agents with pre-eclampsia.

A similar study carried out on 40 women with pre-eclampsia and 27 normotensive women indicated that the trophoblast cells from multiparous women with pre-eclampsia had six times

the risk of being infected with Adeno-associated virus-2 (AAV-2) (OR 6.0; P=0.04). Accordingly the study suggests that AAV virus may destroy the trophoblast cells and would cause an abnormal invasion in the maternal spiral artery (Arechavaleta-Velasco et al., 2006).

Research findings also suggest that infection specific inflammation plays a role in trophoblast complications which leads to defective placentation causing pre-eclampsia. It was noted that pre-eclampsia patients with an infection had an altered balance between proliferation and apoptosis of villous trophoblast cells in the placental tissues (Padmini, 2011), and that trophoblast cell viability and invasion is decreased after the infection in women with pre-eclampsia ($P<0.05$) (Gomez and Parry, 2009). Thus the above studies imply that there is a considerable association of infectious agents with pre-eclampsia which highlights the need for further research, especially at the fetal-maternal interface in the placenta.

Recent investigations suggest that infectious agents directly affect the arterial wall which destroy the cyto-trophoblast cells (Arechavaleta-Velasco et al., 2006) and causes endothelial injury and dysfunction (Redman et al., 1999, Sacks et al., 1998). This further causes atherosis and local inflammation which leads to uteroplacental ischemia. Such mechanisms may explain the way maternal infection is associated with clinical manifestations and the aetiology of pre-eclampsia. Adding to this, recent investigations further demonstrates that inflammation and immune mechanisms triggered by infectious agents could be the origin of the pathogenesis of pre-eclampsia (Xie, 2010, Rustveld et al., 2008).

A study carried out by Ponzetto et al suggested that *Helicobacter pylori* and strains carrying the cytotoxin-associated antigen (CagA gene), may add to the inflammatory mechanisms implicated in the pathogenesis of pre-eclampsia. *H. pylori* sero-positivity frequency was higher in women with pre-eclampsia (51.1%) compared to women with uncomplicated pregnancy (31.9%) (OR, 2.668; 95% CI, 1.084-6.566; P = 0.033). The variation was even higher for CagA sero-positivity (80.9 and 14.9%, respectively) (OR, 26.035; 95% CI, 8.193-82.729; P < 0.001) (Ponzetto et al., 2006).

In a similar study carried out in recent years, direct attention to the fact that 85.7% of women with pre-eclampsia were significantly sero-positive for *H. pylori* compared to healthy pregnant women (42.9%, $P < 0.001$). This study confirmed that persistent and virulent *H. pylori* infections cause or adds to the patho-physiology of pre-eclampsia complicated with fetal growth restriction (Cardaropoli et al., 2011).

Observations made over the years on the occurrence of pre-eclampsia recognized a seasonal pattern of pre-eclampsia similar to diseases like parvovirus and malaria. It is surprising to discover that the occurrence of pre-eclampsia coincide with the seasonal variations of malarial transmission (Arechavaleta-Velasco et al., 2002, Brabin and Johnson, 2005) with a 5.4 fold increase of risk of maternal deaths from pre-eclampsia during such seasons (Anya, 2004).

An animal model of pre-eclampsia developed by introducing low doses of bacterial endotoxin to pregnant mice in their middle gestation developed clinical symptoms similar to pre-

eclampsia. The mice experienced elevated blood pressure, urinary albumin secretion, platelet coagulopathy and histopathological alterations to kidney and placenta (Ponzetto et al., 2006).

Notwithstanding the fact that not many studies have been carried out on this line, a study carried out at Virginia Commonwealth University USA, reports an association of *Sneathia amnii* with pre-eclampsia and other pregnancy related complications. *Sneathia amnii* is a potential bacteria of the female reproductive tract of which very little is known so far (Harwich et al., 2012). Just a couple of years back, *Lactobacillus iners, Ureaplasma / Sneathis leptotrichia* and *Streptococcus* sp. were identified in the amniotic cavity by using the latest 16S rRNA gene amplification and sequencing (DiGiulio et al., 2010).

It is interesting to note that intake of foods rich in probiotics reduce pregnancy related complications associated with pre-eclampsia. A cohort study carried out on 33,399 Norwegian primiparous women in the years 2002 to 2008 reports that probiotic milk products has an association with reduced risk of pre-eclampsia. This could be due to the fact that probiotics modulate gastrointestinal health by suppressing pathogenic bacteria (Brantsaeter et al., 2011).

These studies further supports the position that maternal immune system and the underlying inflammatory level plays an important role in the pathogenesis of pre-eclampsia by providing evidence signifying the association of maternal infections with the increasing risk of pre-eclampsia (Brantsaeter et al., 2011).

In a study carried out by Barak et al in Israel, placental tissue samples obtained from a group of women with pre-eclampsia and a group of matched controls were examined for infectious agents. Placental tissue samples were collected at the time of caesarean section to avoid vaginal and cervical contamination. Samples were examined for infectious agents using traditional culture methods and real time polymerase chain reaction (qRT-PCR) method. 50% (8 out of 16) placental specimens from women with pre-eclampsia were positive for periodontal bacteria compared to 14.3% (2 out of 14) of the controls ($p \leq 0.0055$). Results were statistically significant in the pre–eclampsia group for all the pathogenic bacteria (Barak, 2007). This study supports the role of placental infection in association with pre-eclampsia.

From the review of scientific literature, it is evident that there is a significant association of infectious agents with pre-eclampsia. However, numerous studies detected varying species of infectious agents created controversies on the exact organism(s) responsible for the clinical manifestation of pre-eclampsia. Accordingly, Xie reports that pre-eclampsia is related more closely with total 'infectious burden', than to a specific infectious agent itself (Xie, 2010, Magee and von Dadelszen, 2007).

In addition, the literature supports the view that a single infectious event would be sufficient to trigger the inflammatory response that would lead to pre-eclampsia (Von Dadelszen, 2000, von Dadelszen and Magee, 2002). The above literature strongly suggests that infection may play a key role in the aetiology of pre-eclampsia. Under such circumstances more studies would be essential to further confirm the relationship between pre-eclampsia and infectious agents. Once the relationship is confirmed, new interventions on prevention with non-teratogenic anti-microbial treatment could follow (Todros, 2007).

Accordingly, the current study was based on the hypothesis that pre-eclampsia is caused by one or more infectious organisms present in the placenta, with a predominant organism triggering the condition. It is not possible to obtain the placenta at early gestation due to ethical and technical reasons. The alternative therefore was to examine the placenta of women with preeclampsia, harvested at the time of delivery using culture independent methods. Such methods would enable one to detect traces of infectious agents left behind almost after nine months of pregnancy. 16S rRNA gene sequencing, on Sanger and next generation sequencing platforms (metagenomics) are the highly sensitive genetic techniques that have only now become available. Therefore this study was aimed at detecting and identifying the viruses, bacteria and fungi present in the placental tissues of women with preeclampsia and a control group of normotensive pregnant women.

1.6 Metagenomics used for the detection and identification of 'Infectious Agents'

The use of molecular genetics techniques for the detection and identification of infectious agents has become increasingly popular during the past decade. The technique in comparison to the conventional culture and microscopy methods provides high sensitivity, specification, reproducibility and ease of performance in detection and identification of infectious agents (Pfaller, 2001).

We therefore propose to screen placental tissue samples of women with pre-eclampsia for the presence of viruses, bacteria and fungi using metagenomic techniques. Under 16S Metagenomics, bacteria will be identified from clinical specimens by amplification and sequencing of the 16S ribosomal RNA gene. The resulting sequences are compared against the sequences of all bacteria available in the GenBank database (Petti et al., 2005, Siqueira et al., 2002, Janda and Abbott, 2007, Drancourt et al., 2004, Jordan and Durso, 2000, Millar et al., 1996, Knox et al., 1998, Harris and Hartley, 2003, Whitelaw et al., 2002, Diemert et al., 2002).

28S Metagenomics is performed for the identification of fungi (Ferrer, 2001, Embong, 2008, Viela, 2005, Borneman 2000, Voigt, 1999). Similar to 16S Metagenomics, the 28S ribosomal RNA gene of fungi will be amplified, sequenced and compared against the sequences of all fungi present in the GenBank database. Viruses will be identified using viral metagenomics. Viral DNA will be ligated onto a vector digested with restriction enzymes, transformed into competent cells and will be selected through blue and white colony selection. White colonies are picked up, sequenced and are analysed using BLAST tool (Finkbeiner, 2008, Breitbart, 2005, Delwart, 2009, Edwards, 2005).

1.6.1 16S rRNA gene for the detection of bacteria

1.6.1.1 Introduction to 16S rRNA gene sequencing

Molecular diagnostic techniques such as polymerase chain reaction (PCR) has proved to be more effective in identifying uncultured micro-organisms compared to clinical diagnostic techniques (Fredricks et al., 2007). As of 20th November 2013, the complete genome sequences of 4509 bacteria, 600 fungi and 3810 viruses is present in GenBank database (Siddiqui, 2007). The record covers all known bacteria, fungi and viruses, facilitating the possibility of screening all kinds of clinical specimens for all known bacteria, fungi and viruses.

The conventional mode of detection and identification is based on gram staining, cultures on various media and identification is based on phenotypic observations. However, these conventional techniques usually provide negative results when a patient is on antibiotics, the sample is old or not kept under proper storage conditions. Certain bacteria need specific growth conditions and nutrition. Such bacteria fail to grow on regular growth media and resulting in the possibility of "false negative" results which could lead to misdiagnosis and incorrect treatment.

Broad-range 16S ribosomal RNA gene amplification by PCR (16Sr RNA PCR test) is used for the detection and identification of bacterial agents from clinical specimens of patients suspected of having acquired an infection. The technique works with optimal sensitivity and provides specific results for patients on antibiotics (Rampini et al., 2011). Bacterial 16S rRNA contains highly conserved nucleotide regions common to all bacterial species and variable nucleotide regions unique at species level. With the use of universal primers partial or the

complete 16S rRNA gene is amplified and the bacteria present in the clinical specimen or an environmental sample is easily identified.

The amplified 16S ribosomal RNA was sequenced either by capillary or next generation sequencing. The sequences obtained was blasted against all bacterial sequences present in online bacterial databases such as GenBank, Ribosomal, Greengenes databases facilitating specific identification of the bacteria present in the respective sample (Drancourt et al., 2004, Janda and Abbott, 2007, Jordan and Durso, 2000). The approach is extremely useful in distinguishing new sequences of formerly uncharacterized bacteria and has enabled the identification of many new strains within the last few years. 16S rRNA gene sequences play an important and major role in discovering bacterial phylogeny and taxonomy due to its presence in more or less all bacteria (Janda and Abbott, 2007). The properties of the gene have not undergone any major evolutionary changes and had remained unaltered over the years. 16S ribosomal RNA gene is approximately 1500bp and is adequate enough to obtain the required information for research and study purposes.

Another major striking feature of 16S rRNA gene sequencing is its capability of identifying an organism up to genus and species level, even when it does not fit any possible biochemical profile. 16S rRNA gene sequencing is an approach widely used as an alternative when conventional biochemical profiling and culture based techniques fail to identify a particular strain.

It is evident from research studies that the said technique has the capability to detect and identify non-viable organisms which remain undetected under traditional methods. The approach is also useful for the identification of slow growing fastidious organisms where traditional isolation methods experience difficulties. The traditional techniques are time consuming and growth of bacteria may take days (Drancourt et al., 2000). However, 16S rRNA PCR test and sequencing enables the identification of bacteria within a few hours. Hence 16S rRNA based testing surpasses the conventional methods so far used (Janda and Abbott, 2007). Previous studies revealed that 16S rRNA gene sequencing is capable of detecting 90% of organisms up to genus level, 65-83% up to species level and only 1-14% isolates remained unidentified using the 16S Metagenomics technique (Janda and Abbott, 2007, Drancourt et al., 2000).

Universal distribution of 16S rRNA gene within the bacterial population plays a significant role in the molecular identification of the organism (Vetrovsky and Baldrian, 2013). The presence of species specific variable regions in the 16S ribosomal RNA gene of bacteria helps in the specific identification and demarcation (Drancourt et al., 2000). It has been evident over the years that conventional techniques in identifying bacteria most often failed due to inadequate knowledge on growth requirement of the isolates, faults in morphological identification and failure of gram staining. In addition, the viable state of organisms play a vital role in culture based conventional methods of identification. 16S rRNA gene based technology provides a solution to such difficulties and has been shown to be an excellent performer in identifying over 90% of bacteria within a larger population of phenotypically unidentified bacteria (Drancourt et al., 2000).

1.6.1.2 16S ribosomal Ribo-Nucleic Acid (16S rRNA) gene

Ribosomal Ribo-Nucleic Acid (rRNA) is the RNA constituent of the ribosome which plays a vital role in protein synthesis in all living beings. The size of prokaryotic ribosome is 70S (S represents Svedberg units) which is made up of a large sub unit of 50S and a small subunit of 30S. 5S ribosomal RNA and 23S ribosomal RNA are components of the 50S large subunit and the 16S ribosomal RNA is the component of the 30S small subunit of the ribosome. Much attention is focused on the 16S rRNA gene which has remained the gene of choice for sequencing and reconstructing phylogenies for numerous reasons. It needs to be mentioned that all credits pertaining to this amazing technological discovery belongs to Carl Woese and George E. Fox (Fox et al., 1977, Woese and Fox, 1977). As mentioned previously it is a highly conserved gene which has not undergone any major evolutionary changes over the years. The minor changes that have taken place offers an indication as to how closely or distantly the diverse organisms are associated.

This gene which is 1.5 kb in size is made up of both conserved and variable regions with inter specific polymorphisms capable of distinguishing between species (Clarridge, 2004). As illustrated in Figure 1.7, the 16S rRNA gene has 9 variable regions where partial sequencing of 3 consecutively variable regions is considered the most useful approach. Combinations of V7, V8, V9 and V1, V2, V3 are considered to be the most sensitive and specific for the detection and identification of bacteria up to species level (Cai et al., 2003).

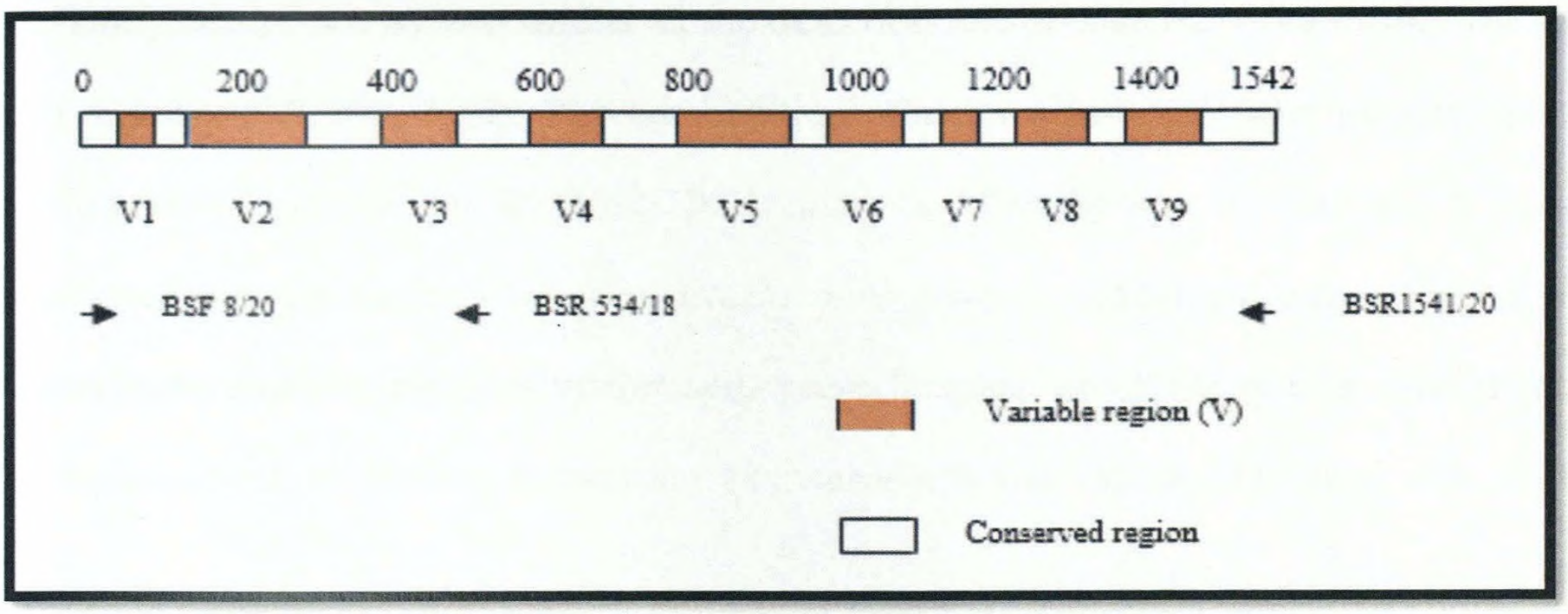


Figure 1.7 16S rRNA gene and sequencing primer locations

16S rRNA gene is unique to all bacteria and is 1542bp in length with 9 variable regions

The variable regions within the 16S rRNA gene are subjected to variations which provide the necessary diversification essential for differential classification. The conserved regions are also equally important as they assist in designing of suitable primers and hybridization of probes even at different taxonomic levels (Vetrovsky and Baldrian, 2013).

1.6.1.3 Pre-eclampsia studies carried out using 16S rRNA sequencing technology

Previous studies using conventional methods suggest an association of infections with pre-eclampsia (DiGiulio et al., 2010). A study carried out in USA in 2010 remains the only study to date which has employed 16S gene amplification to investigate the microbial invasion of the amniotic cavity in pre-eclampsia (DiGiulio et al., 2010). Accordingly the current study remains the only study that used 16S metagenomic technique to detect and identify bacteria in the placental tissues of women with pre-eclampsia.

In the study carried out by DiGiulio et al, the detection rate of bacteria by culturing was 1.6% (1/62) and by PCR 8% (5/62). The combination of culture and molecular genetics gave a detection rate of bacteria at 9.6% (6/62). Respiratory and food borne infectious agents such as *Ureaplasma parvum, Escherichia coli, Listeria monocytogens* and *Streptococcus* species were detected in the amniotic cavity of women with pre-eclampsia, which suggests an association of pre-eclampsia with food borne & respiratory bacteria/ infectious agents (DiGiulio et al., 2010).

1.6.2 28S rRNA gene for the detection of fungi

1.6.2.1 Introduction to 28S rRNA gene sequencing

Amplification of 28S ribosomal RNA gene for the detection of fungi is a novel technique that has become increasingly popular in the past few years. Quick detection and precise identification of fungal agents is an utmost necessity for a precise antifungal therapy (Khot et al., 2009b). The conventional culture-based and histological analyses often results in poor diagnostic sensitivity and fails to differentiate between fungal species and very often remains undetected (Yeo and Wong, 2002, Khot et al., 2009b).

28S metagenomics for the detection of fungal agents is the most rapid, sensitive and accurate diagnostic approach. The 28S metagenomics is based on the principle that the 28S ribosomal RNA gene has conserved regions for designing broad-range primers and also variable regions for the differentiation of fungal species. The 5' end of the large subunit which is the D1-D2 hyper-variable region in the 28S rRNA gene is most often targeted for the identification of human fungal agents (Evertsson et al., 2000, Hinrikson et al., 2005, Kurtzman and Robnett,

1997, Rakeman et al., 2005, Vollmer et al., 2008, Putignani et al., 2008, Khot and Fredricks, 2009a, Khot et al., 2009b).

1.6.2.2 28S ribosomal Ribo Nucleic Acid (28S rRNA) gene

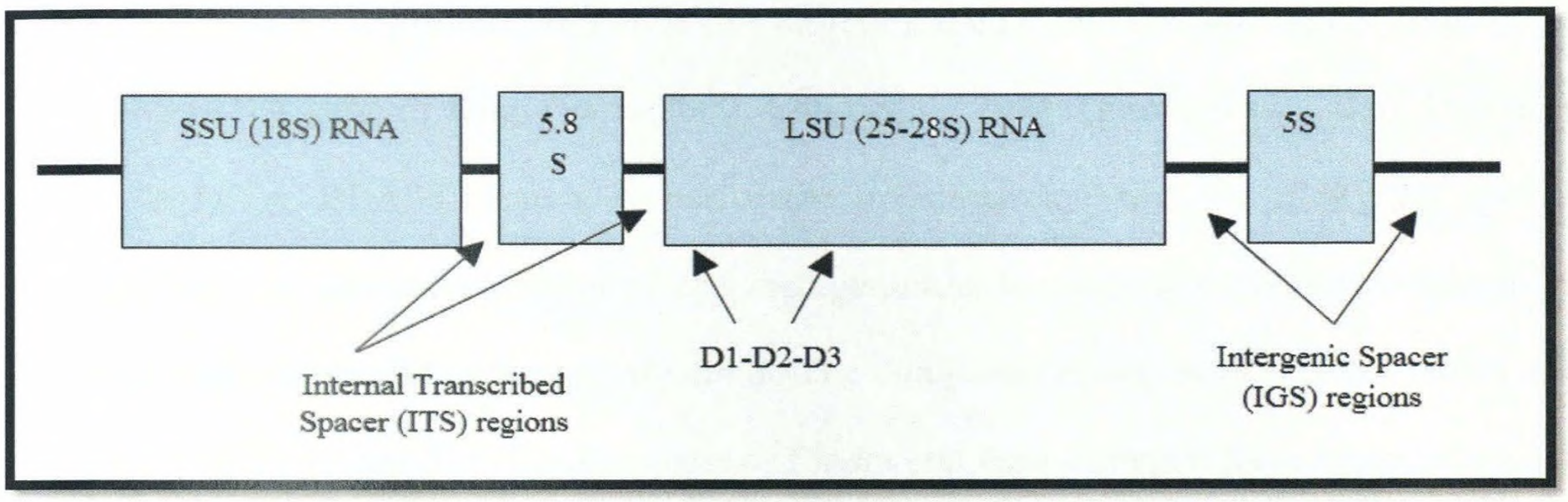


Figure 1.8 28S rRNA gene of fungi

28S rRNA gene is unique to all fungi and is 4kbp in size. However, D1, D2, D3 regions are the most variable regions and is sufficient for species level identification

The 18S rRNA gene is 1995bp in length whilst the 28S rRNA gene varies in length from 3948 to 3976bp (Stage and Eickbush, 2007). Most studies carried out so far only utilized the first 600-900 bases from the large sub unit of 28S rRNA gene, which includes three divergent domains (D1, D2, and D3) that are the most variable region within the entire gene. There is nearly a 2900bp variable region beyond the D2 region which still remains unexploited. Therefore amplifying a region beyond the D1-D2 region under present circumstances may be less useful for species identification (Khot et al., 2009b).

The 28S ribosomal RNA of fungi is about 4kbp in size and therefore, the whole genome sequencing of the 28S rRNA is extremely costly and laborious (Park, 2002). However,

amplifying the D1- D2 variable region is sufficient for the identification of fungi at species level from clinical samples (Park, 2002, Khot and Fredricks, 2009a, Khot et al., 2009b) and can be considered the best available diagnostic tool for the identification of fungi.

Many studies use PCR primers NL1 and NL4 targeting the D1 and D2 domains to amplify the partial 28S rRNA gene of fungi (Park, 2002, Cano et al., 2004, Garner et al., 2010, Lachance et al., 2003). A BLAST search is performed to determine the specificity of species identification. The detection of fungi by 28S metagenomics has been described as an important tool in the early diagnosis of fungal infections. The complete sequences of the 28S rRNA and a large number of partial 28S rRNA sequences for several fungal species have being published in GenBank database (Vollmer et al., 2008). As at 20th November 2013, there are 600 whole genome of fungal species available at the NCBI GenBank database (Siddiqui, 2007). In the present study the 28S metagenomics is used by amplifying a region within the D1-D2 variable region of the 28S rRNA gene for the detection of fungi in the heterogeneous human clinical specimens.

1.6.2.3 Pre-eclampsia studies carried out using 28S rRNA gene sequencing

Studies carried to determine the association of fungal infections with pre-eclampsia is inadequate. Consequently, the current study will be the first study carried out using 28S metagenomics to investigate the association of fungi present in the placental tissues of women with pre-eclampsia.

1.6.3 Sanger/ Capillary sequencing

Positive PCR products confirmed for 16S/28S rRNA gene were subjected to 'automated sequencing'. This enabled the identification of the organism present in the amplified positive samples. Automated DNA sequencing allows the determination of the exact nucleic acids (order of Adenine, Cytosine, Thymine and Guanine) in a DNA molecule or the amplified gene of interest. The main principle behind the automated sequencing was the 'Sanger Chain Termination' chemistry. This was carried out using the PCR product as the template in a cycle sequencing reaction which facilitates the integration of fluorescence labelled dideoxy nucleotides (ddNTPs) into the sequencing product which was detected by semi-automated capillary electrophoresis. This method would read up to 700-1000bp of a DNA segment (Wallis, 2011).

Before the cycle sequencing reaction was prepared it is necessary to have a purified PCR product. This was done with the treatment of Exo-SAP cleanup process. Exonuclease 1 (Exo1) was used to remove the excess primers in the PCR mixture and Shrimp Alkaline Phosphatase (SAP) was used to remove the additional deoxy nucleotide tri phosphate (dNTPs). Cycle sequencing was carried out with the ABI PRISM Big Dye® terminator v3.1 cycle sequencing kit (Applied Biosystems, USA). The kit contained fluorescent dye–tagged terminators which were four dNTPs each labelled with a fluorescent tag. The main principle of this kit was based on the Sanger's chain termination dideoxy nucleotide method of sequencing theory (Sanger et al., 1977). At the end of the cycle sequencing reaction PCR products of varying sizes terminate by adding a fluorescently labelled ddNTP. This would be read automatically by the capillary electrophoresis in the ABI PRISM 3130 Big Bye® Genetic Analyzer (Applied Biosystems, USA) and would result in a coloured electrophorogram.

1.6.4 PCR product properties for thermo-stable DNA polymerase

The thermo-stable DNA polymerase used for PCR has a great effect on the cloning experiment. It is because not all thermo-stable polymerases produce the PCR fragment with 3'A-tailed. Some proofreading polymerases such as *Pfu* DNA polymerase, *Pwo* DNA polymerase and *Tli* DNA polymerase produce blunt–ended PCR fragments. The A tailed insert DNA fragment would directly ligated to the T tailed plasmid vector with the action of T4 DNA ligase to be recombined. However as indicated in the figure 1.9 Go Taq® DNA polymerase, Ampli Taq® polymerase, Tfl & Tth DNA polymerases produces 3'A overhang PCR fragments (Promega, 2010). Therefore all amplifications carried out using polymerase chain reaction performed prior to the cloning experiment were carried out using Go Taq® DNA polymerases (Promega, USA).

Figure 1.9 Comparison of PCR product properties for thermo stable DNA polymerases

Characteristic	Thermostable DNA Polymerase						
	GoTaq®/ *Taq*/ AmpliTaq®	*Tfl*	*Tth*	Vent® (*Tli*)	Deep Vent®	*Pfu*	*Pwo*
Resulting DNA ends	3´A	3´A	3´A	Blunt	Blunt	Blunt	Blunt
5´→3´ exonuclease activity	Yes	Yes	Yes	No	No	No	No
3´→5´ exonuclease activity	No	No	No	Yes	Yes	Yes	Yes

(Promega, 2010)

1.6.5 Cloning of target DNA using pGEM T vector

When amplified PCR product results in a distorted electrophorogram due to a mixed infection it is not possible to identify the bacteria present in the sample. In such instances the amplified 16S rRNA gene needs to be cloned. By sequencing individual colonies it would be possible to identify the different bacteria present in the sample. This approach was one of the most flexible and effective ways of screening for bacteria using the meta-genomics library which has the sequencing data for almost all bacteria (Wexler and Johnston, 2010, Handelsman, 2004, Wooley et al., 2010).

After the purification of the amplified DNA, it was cloned into a pGEM®- T vector. Cloning is a laboratory procedure to produce similar copies of DNA fragments. The pGEM®- T vector is a linearized vector and has single 3' terminal thymidine at both ends. The PCR product ligates to the T- overhangs at the insertion site and prevents the re-circularization of the vector. The T over hangs at the insertion site facilitate an efficient ligation of the PCR product (Promega, 2010). Promega's pGEM®-T Vector systems are designed for most general cloning applications. The pGEM®-T Vector is a high-copy number plasmid which has T7 and SP6 RNA polymerase promoters flanking the multiple cloning region. The promoters help to sequence the insert as well as for in vitro transcription of both sense and anti-sense RNAs. The multiple cloning region of the vector is present within the α-peptide coding region of the enzyme β-galactosidase to facilitate the blue/white screening. A variety of restriction sites are available within the vector for sub cloning, and contains a BstZ I restriction site for the release of a cloned insert by single restriction digestion (Bassetti, 2002).

The pGEM® - T vector was developed from the pGEM® - 5Zf(+) vector (GenBank® Accession No.X65308) and was produced by linearizing the pGEM® - 5Zf(+) vector with EcoRV at base 51 and adding a T to both 3' ends as indicated in Figure 1.11. The EcoRV site would not be recovered upon ligation of the vector and insert (Promega, 2010). Figure 1.10 indicates the pGEM® - T vector map and sequence reference points. Thus inserts can be sequenced using the SP6 promoter primer, T7 promoter primer, PUC/M13 forward and reverse primers (Promega, 2010).

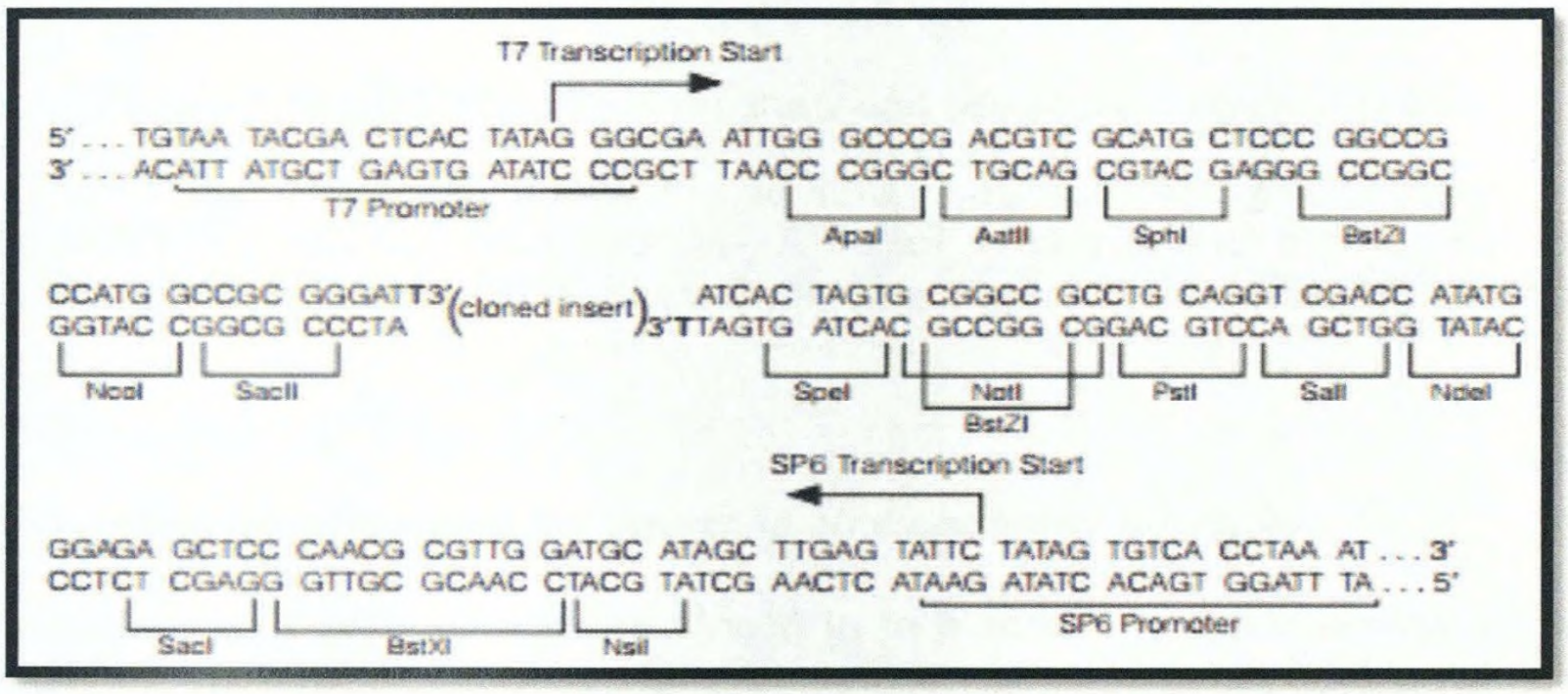


Figure 1.10 The promoter and multiple cloning sequence of the pGEM®-T vector

The top strand corresponds to the RNA synthesized by T7 RNA polymerase. The bottom strand corresponds to the RNA synthesized by SP6 RNA polymerase (Promega, 2010)

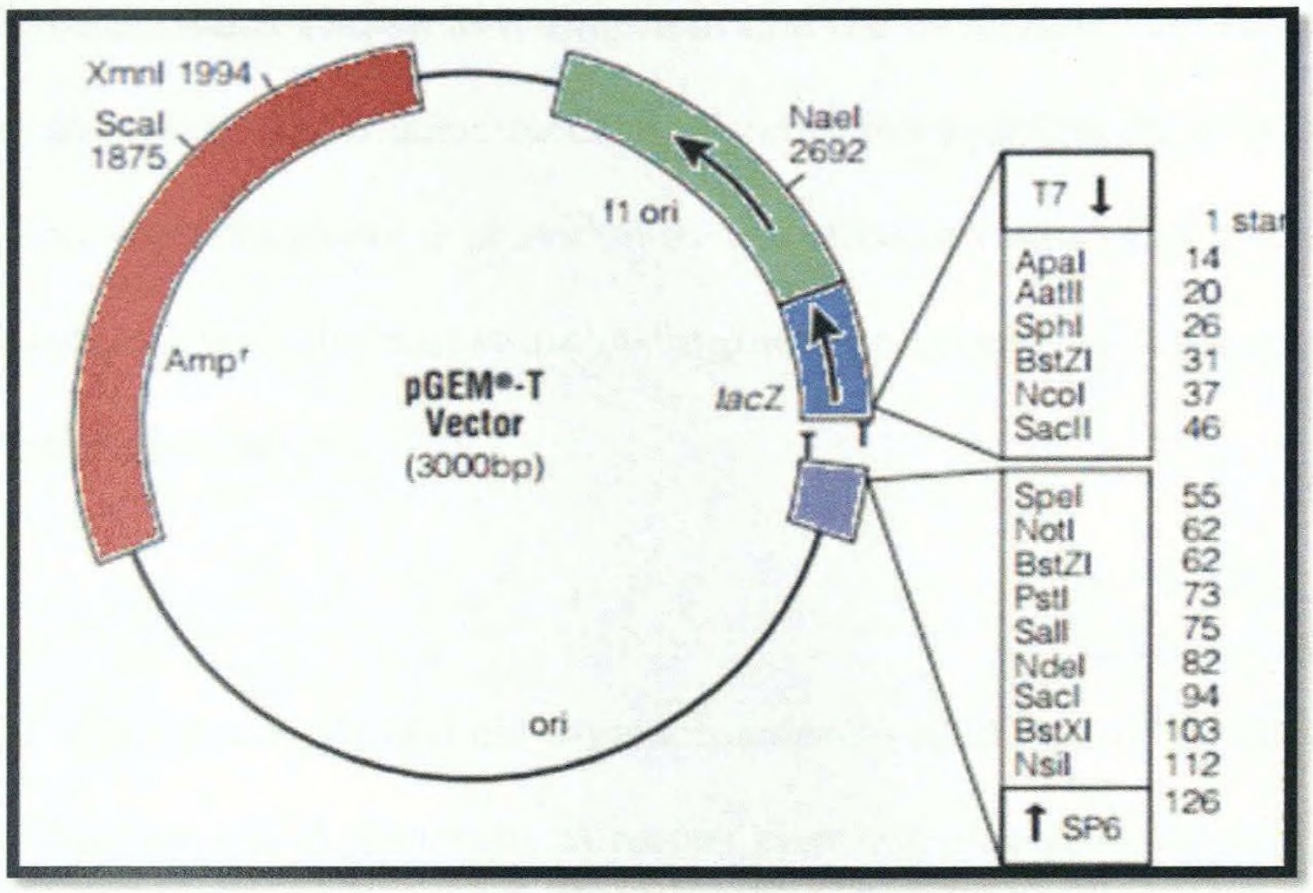


Figure 1.11 pGEM®- T vector map and sequence reference points

pGEM- T is a plasmid vector of 3000bp in size. It has Amp^r and lac Z genes. It was developed from the pGEM® - 5Zf(+) vector. pGEM- T vector is compatible with many restriction enzymes. T7 and SP6 primers will facilitate the sequencing of the insert DNA (Promega, 2010)

1.6.5.1 Screening transformants for inserts by blue and white selection

Once the target insert gets successfully cloned in to pGEM®- T vector it would disrupt the coding sequence of β-galactosidase and hence the recombined clones could be identified by colour screening on the indicator plates. The colonies with the insert produces white colour colonies and the blue colonies are colonies without the insert. Occasionally blue colonies too may contain the target insert if it had cloned in frame with the *lac*Z gene. However, with longer incubation time blue colour deepens and can be easily differentiated.

The pGEM®- T vectors are high copy number vectors containing T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the α-peptide coding region of the enzyme β-galactosidase. The enzyme β-galactosidase is a product of the bacterial *lac*Z gene. It is

separated on to two domains known as α-fragment and the ω-fragment. These two domains act together to form the functional β-galactosidase. Usually the ω-fragment is expressed by the *E. coli* host strain and the α-fragment is provided by the cloning vector. Once intact in frame, the α-fragment will interact with the host strain ω-fragment and create functioning β-galactosidase known as 'α- complementation'.

Bacteria capable of producing functional β-galactosidase would cleave the substrate X- Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) creating blue colonies when grown on an indicator plate containing IPTG/X-Gal (Promega, 2013a). However when the sequence of interest (Insert DNA fragment) was inserted within this region, inactivation of the α-peptide prevented 'α-complementation' and produced white colonies. This facilitated the identification of recombinants by blue/white screening on indicator plates (Promega, 2010).

1.6.6 Viral Metagenomics technique

1.6.3.1 Introduction to viral metagenomics technique

The detection of new viruses often becomes hindered by difficulties in amplifying them in cell culture, due to limitations in antigenic/serologic cross reactivity or the lack of nucleic acid hybridization to known viral sequences (Delwart, 2007). 99% of viral infectious agents are uncultivable and cannot be detected using conventional methods (Riesenfeld et al., 2004, Angly et al., 2009). In addition there isn't a single gene (universal gene) that is common to all viruses due to the higher level of genetic modifications viruses undergo. Thus the total uncultured viral diversity cannot be monitored using approaches corresponding to ribosomal DNA profiling (Edwards, 2005).

In the latest metagenomic studies, viral particles from uncultured environmental and clinical samples have been purified and their nucleic acids randomly amplified prior to sub cloning and sequencing. Both recognized and novel viruses were then identified by comparing the sequences to the viral sequences present in public sequence databases (Delwart, 2007). There are 3810 viral whole genome sequences currently available at the NCBI GenBank database (Siddiqui, 2007).

Viral Metagenomics technique for the classification of viruses have been used for environmental samples such as seawater, near shore sediments, soil and clinical specimens such as faeces, serum, plasma and respiratory secretions. This has contributed extensively to the understanding of viral diversity (Delwart, 2007). Viral metagenomics has opened up the doors for the detection and identification of viruses from natural habitats and biological

samples independent of PCR amplification and allows an unbiased classification of viruses. Viral Metagenomics is a powerful tool that can be incorporated into the medical sector to study uncultured viruses from biological samples where ordinary PCR assays fail to detect viruses (Svraka et al., 2010).

Conventional methods of viral diagnostics use primers targeting the amplification of the more likely viral groups that may be present in the sample. The technique is capable of detecting only already known viral types and the presence of a new viral strain remains undetected. The most prominent character of a virus is the rapid rate it undergoes mutations and its ability to form new viruses in comparison to other organisms (Sanjuan et al., 2010). Viruses, particularly RNA viruses, mutate at a very high rate per genome per replication (Regoes et al., 2013, Drake and Holland, 1999). There is therefore a clear need for having a more precise technique which can characterize all or any kind of viruses present in the sample even when they are previously unrecognized.

Viral Metagenomics is the answer for the detection and the identification of viruses with a tendency for higher rate of mutations to form new strains. The approach focused on sequence-independent amplification (whole genome amplification), sub cloning and sequencing of purified viral nucleic acids followed by BLAST search for similar sequences to identify the specific viruses from an environmental sample or fresh biological sample, is known as 'Viral Metagenomics' (Delwart, 2007). Viruses extracted from human or an animal host is often limited by the amount of viral DNA available. The properties of PhiX29 polymerase make it possible to amplify the entire human genome, starting from as little as 10 cells, until 20–30mg

of DNA are isothermally produced. Use of PhiX29 DNA polymerase based amplification for viral discovery is becoming increasingly popular now a days (Delwart, 2007).

The Illustra™ GenomiPhi™ V2 and HY DNA amplification kits are the latest additions to the Phi 29 DNA polymerase family of products from GE Healthcare. GenomiPhi V2 DNA amplification kit yields 4–7 μg of representative genomic DNA (gDNA) in 1.5 h from 1–10 ng of input genomic DNA (Kumar, 2007). For better identification efficiency larger amount of viral nucleic acids and less background prokaryotic and eukaryotic nucleic acids is an essential requisite. Most researchers recommend the removal of background host DNA contaminations by filtration using filters with pore size as small as 35nm. Alternatively treatment with DNAase1 and RNAase 1 removes naked DNA and RNA leaving behind the viral DNA within the viral capsids (Delwart, 2007). The purified viral DNA would be whole genome amplified, digested with restriction enzymes, sub cloned and sequenced. By BLAST option available at the NCBI database FASTA sequences obtained is aligned to match with the viral sequences available at the GenBank database. As viral metagenomics is an emerging field with a high scope of detection and identification capabilities of viruses from clinical specimens it is the methodology adopted in this research project.

1.6.3.2 Studies of pre-eclampsia carried out using viral metagenomics

To our knowledge, no study has been carried out to detect and identify viruses present in biological samples of women with pre-eclampsia using viral metagenomics. Accordingly, the current research project remains the first study to employ viral metagenomics to detect and identify viruses in the placental tissues of women with pre-eclampsia.

1.6.7 Metagenomics using Sanger sequencing

The science of metagenomics is a recent discovery and has facilitated the investigation of microbes in their natural environment itself. It is a discipline that enables the genomic study of uncultured microorganisms using molecular genetic techniques. Metagenomics is the microbial analysis tool with the best resolution and analyse genomic DNA from a whole community (Prathapan, 2012, Attygalle, 2011). Understanding the microbial diversity and function in a community can lead to the discovery of novel molecules for therapeutics and biotechnology applications.

To protect human health it is necessary to develop and use methods which detect all microbes present in a specific environment. Metagenomics have opened up the doors to study the microbiome in biological specimens. Microbiome is the totality of microbes, their genetic elements (genomes) & environmental interactions in a particular environment. Advancements in sequencing technology have enabled the availability of the whole genome sequences of a majority of clinically relevant micro-organisms. This brought other decisive aspects to the field of metagenomics by discovering a vast majority of microorganisms which had previously remained unnoticed (Riesenfeld et al., 2004, WHO, 2012).

Metagenomics based on Sanger sequencing is a method adapted prior to next generation sequencing era. It is time consuming, due to the many steps involved, quite arduous and expensive. Sanger sequencing, results in non-quantitative limited amount of data compared to next generation sequencing (Liu et al., 2012). Metagenomics based on Sanger sequencing requires the sub cloning of the PCR amplified template, sub cloning, picking up colonies with the insert, sub culturing and sequencing the clones using capillary electrophoresis (Sanger

sequencing) to identify bacteria, fungi and viruses present in the biological samples of women with pre-eclampsia.

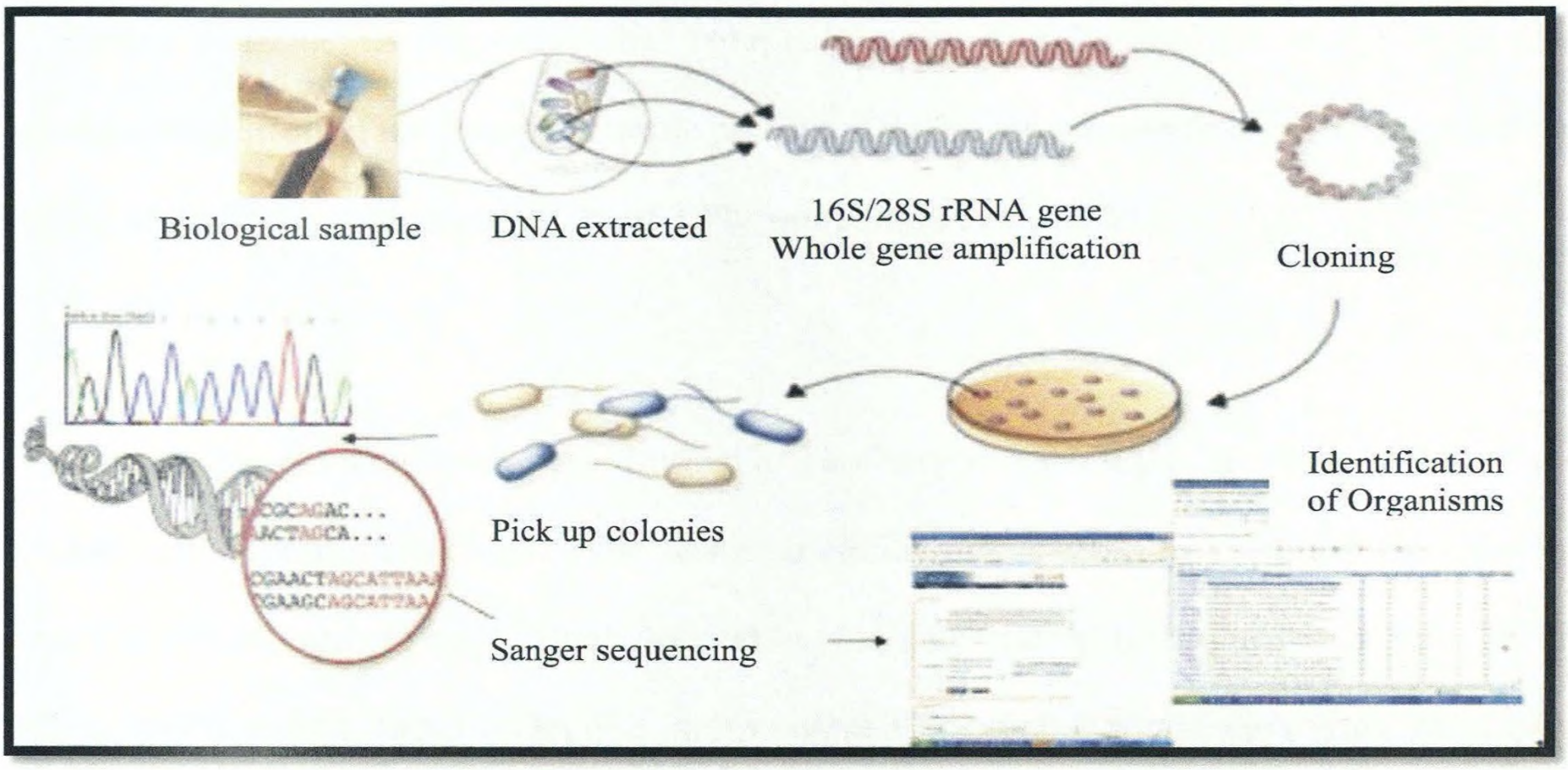


Figure 1.12 Metagenomics based on Sanger sequencing

Metagenomics based on Sanger sequencing involves the amplification of 16S rRNA gene followed by cloning and Sanger sequencing the individual colonies for the identification of bacteria.

By cloning 10 colonies per sample, obtaining a full picture of the bacterial population (microbiome) present in the particular sample would not be a possibility. In contrast the next generation sequencing technique has overcome these limitations and analyses high through put data. It can identify bacteria in a mixed population without the need for cloning or culturing. Therefor it is a convenient, economical, fast and accurate technique (Illumina, 2012b). Next generation sequencing technique is the latest addition to the field of metagenomics.

1.7 Metagenomics using Next Generation Sequencing

1.7.1 Introduction to Next Generation Sequencing

During the past few decades novel techniques have been discovered to decipher the genetic code. Sanger sequencing played a vital role in genetic diagnostics and was behind the successful completion of the human genome project. The Sanger sequencing method is capable of reading short DNA sequences of about 1000 bp (Zhang et al., 2011).

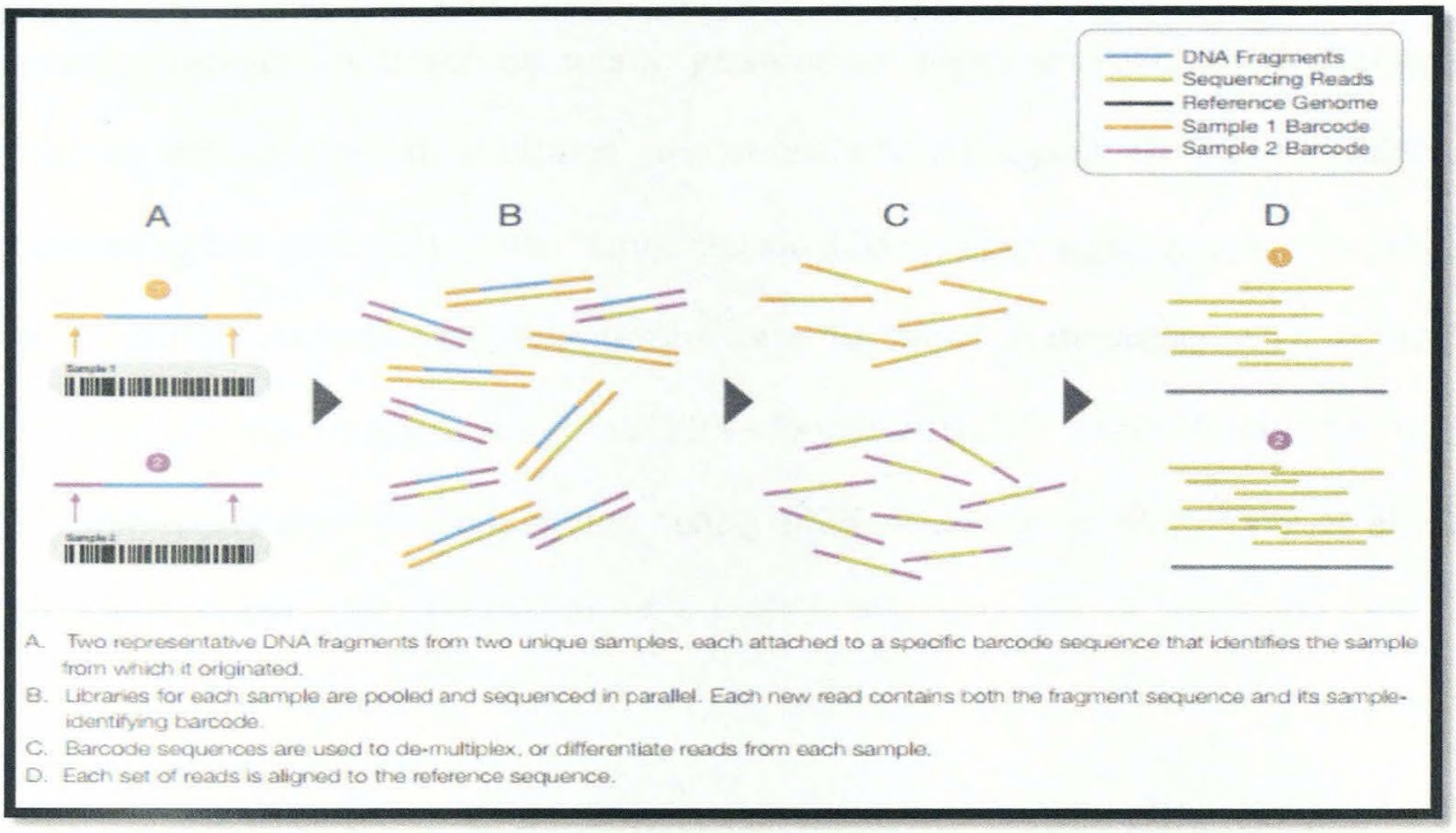
The automated Sanger sequencing also known as capillary sequencing is considered the 'first-generation sequencing technology'. The newer methods are referred to as 'Next Generation Sequencing (NGS) technology' which has taken the sequencing technique to a new level (Metzker, 2009). It is a rapid technique comparatively inexpensive and generates an ample amount of sequencing data (Illumina, 2012b, Metzker, 2009). Its ability to sequence the whole genome of many allied organisms facilitates the execution of comparative and evolutionary studies, unimagined a few years back (Metzker, 2009). The technique leads to the roots of many genetic disorders and will help in understanding the underlying genetic makeup of many diseases such as pre-eclampsia.

The dawn of NGS technology 5 years back revolutionized the field of genetics by providing considerable amounts of genetic information. This has led to many significant discoveries and advancements in the fields of molecular diagnostics of human diseases, agriculture and evolutionary sciences (Illumina, 2012b). Next generation sequencing technology sequentially identify the nucleotide bases of small fragments of DNA and allows rapid sequencing of large sections of DNA covering the entire genome. This cutting-edge instrument is capable of

generating hundreds of giga-bases of genetic data from a single sequencing run (Illumina, 2012b).

In the next generation sequencing technology a single genomic DNA (gDNA) is fragmented into a library of small segments. This library is then consistently and accurately sequenced in millions of parallel reactions. The newly identified strings of bases called reads, are automatically blasted against a known reference genome to find the complete microbiome. In the absence of a reference genome it is considered a novel strain and is known as de novo sequencing. Interestingly, the full set of aligned reads reveal the complete sequence of each chromosome of the genomic DNA (Illumina, 2012b).

NGS technology is highly scalable and can be used to generate high throughput data for larger genomes and lower output volumes for targeted studies or smaller genomes. The scalability access the opportunity of sequencing small bacterial/viral genomes or specific regions like exomes. In addition, multiplexing allows large sample numbers to be simultaneously sequenced during a single experimental run. This is achieved by "barcode" sequences that are added to each sample to differentiate between the samples for data analysis (Illumina, 2012b), as illustrated in Figure 1.13 below.



A. Two representative DNA fragments from two unique samples, each attached to a specific barcode sequence that identifies the sample from which it originated.
B. Libraries for each sample are pooled and sequenced in parallel. Each new read contains both the fragment sequence and its sample-identifying barcode.
C. Barcode sequences are used to de-multiplex, or differentiate reads from each sample.
D. Each set of reads is aligned to the reference sequence.

Figure 1.13 Sample multiplexing with the help of 'Barcode sequencing'

Two representative DNA fragments from two unique samples, each attached to a specific barcode sequence that identifies the sample from which it originated. Libraries for each sample are pooled and sequenced in parallel. Each new read contains both the fragment sequence and its sample identifying barcode (Illumina, 2012b)

To process hundreds of amplicon using Sanger sequencing requires several weeks or months under normal circumstances. In contrast, the same number of samples could be sequenced in a matter of hours and can completely be analysed within two days using the NGS technique (Illumina, 2012b). Another important application of next generation sequencing is its ability to identify bacteria up to species level even in the presence of a mixed infection. This method is widely used to study phylogeny, taxonomy and to evaluate bacterial diversity in a number of different environments. The technique gives out complete microbiome of samples that are otherwise difficult or impossible to analyse (Illumina, 2012b, Mardis, 2008).

1.7.2 16S Metagenomics using Next Generation Sequencing

Meta-genomics stratagem is based on whole genome shot gun sequencing of nucleic acids purified from an environmental or clinical specimen. Over the past 10 years metagenomic shotgun sequencing has gradually shifted from classical Sanger sequencing technology to NGS (Thomas et al., 2012). Accordingly, the current trend of bacterial metagenomics technique is the amplification of whole genome of the 16S ribosomal RNA gene of bacteria by PCR followed by massively parallel sequencing using NGS technology (Petrosino et al., 2009, Jones, 2009, Barzon et al., 2012).

'Metagenomics' is the study of 'metagenome's or genetic materials isolated from microbial communities within their natural habitat itself. As mentioned previously, metagenomics depends on prokaryotic 16S ribosomal RNA gene which is approximately 1500bp with nine scattered conserved and variable regions within the gene. This facilitates sequencing and phylogenic classification up to species level for mixed microbial populations (Illumina, 2013b).

Genetic studies of microbial communities using 16S metagenomics NGS has led to a broader understanding of human health, disease susceptibilities, pathophysiology of infectious and immune mediated diseases (Petrosino et al., 2009). The most important aspect of these platforms is the ability to determine the complete genetic makeup from a single DNA fragment of a library that is segregated on chips, eliminating the need for cloning or culturing prior to sequencing (Nowrousian, 2010, Barzon et al., 2012).

The use of next-generation sequencing technique for metagenomics results in receiving a large amount of sequence data which are derived from numerous environments, such as soil, ocean water and the human body. Analysis of such data has opened up a corridor into the vast taxonomic and functional diversity of environmental microbial communities. The combination of DNA, mRNA and protein based studies of microbial communities present in different environments is the way to interpret the structure, function and relation of microbial communities within an environment (Simon and Daniel, 2011).

Recent studies report that metagenomic analysis is the key to discover mixed infections. With the increasing read lengths and decreasing cost, the latest bench top sequencers like 'Next Generation Personal Genome' machines might finally be used in routine diagnostic laboratories (Junemann et al., 2012). Ion Torrent and Illumina personal genome machines facilitate 16S metagenomics to discover many bacteria from a heterogeneous clinical specimen with the finest resolution (Junemann et al., 2012, Whiteley et al., 2012, Claesson et al., 2010, Thomas et al., 2012).

The introduction of next-generation sequencing platforms such as the Roche 454 sequencer, the SOLiD system of Applied Bio-systems and the Genome Analyser of Illumina had greatly influenced metagenomic research and has resulted in many studies exploring the taxonomic and functional biodiversity in countless ecosystems (Simon and Daniel, 2011). In the future, 16S metagenomics could be used as a finger printing method to describe microbial community profiles (Thomas et al., 2012).

However, according to Simon and Daniel, a complication that exist with the metagenomic technique is that certain sequences may remain unidentified due to the lack of a reference sequence in the GenBank database (Simon and Daniel, 2011). However it is unlikely that the limitation will be a problem for long with the rapid development observed in the field of metagenomics. Already sequences of 16S rRNA gene along with its variable and conserved regions have been determined for a larger number of organisms. These sequences are available in multiple databases such as 'NCBI Genbank', 'Greengenes' and 'Ribosomal Database Project' (Illumina, 2013b). Literature suggests that there are no other studies conducted in the lines of the current research project. This will be the first time bacterial metagenomics using Illumina MiSeq next generation sequencing technique will be conducted for the detection and identification of infectious bacteria in association with pre-eclampsia.

1.7.3 Illumina MiSeq personal sequencing system

Illumina MiSeq personal sequencing system is one of the most accurate and easy to use bench top genetic analysers. The optimized sample preparation kit, push button sequencing and automated data analysis has made it possible to sequence a sample within two days. With the availability of 'Base Space' option it is possible to store, analyse and share data without the need of additional hardware making it very cost effective and simple. MiSeq sequencing workflow consists of four main steps;

- Library Preparation
- Cluster Generation
- Sequencing
- Data Analysis

Sample preparation is critical for successful sequencing; therefore the aim of the sample preparation step is to obtain nucleic acid fragments with adapters attached on both ends as shown in Figure 1. 14 below

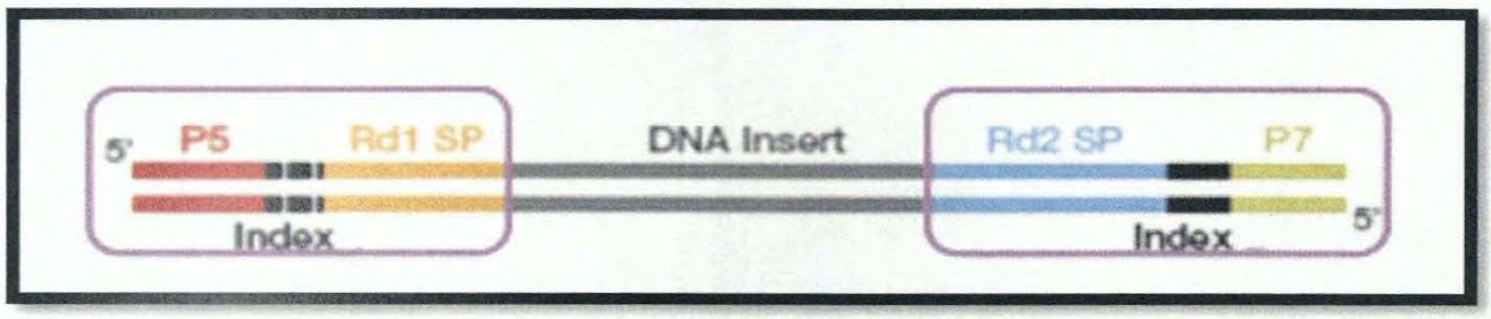


Figure 1.14 Dual index library

Target DNA insert flanked with two indexes (adapters) (Illumina, 2013b)

Samples are prepared using the 'Nextera sample preparation kit' which uses transposomes to fragment the DNA and to add adapters. This kit does not require separate fragmentation and is tagged with enzyme mix as shown in Figure 1.15.

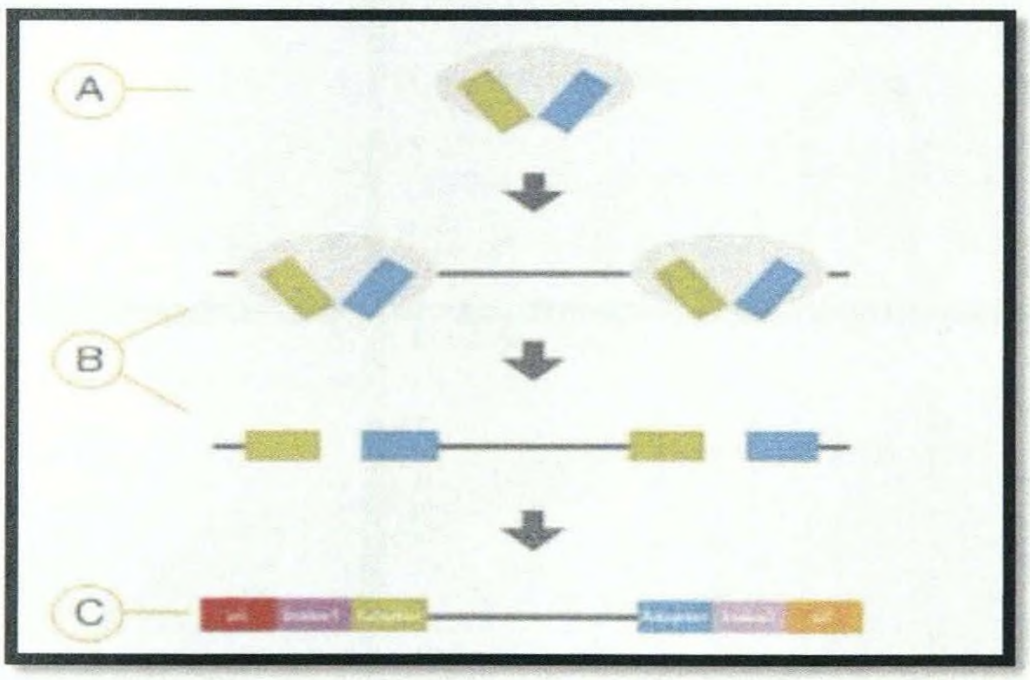
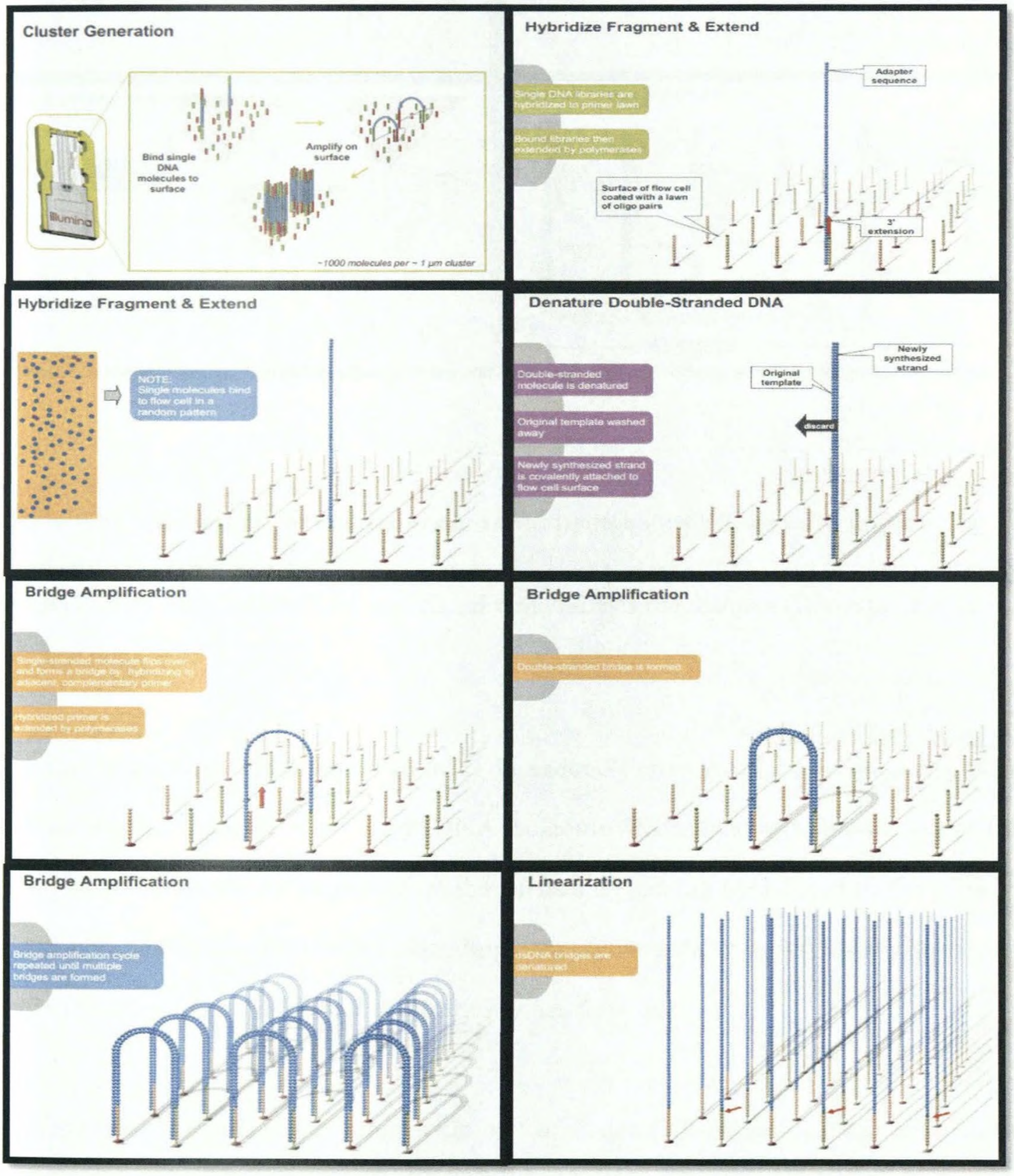
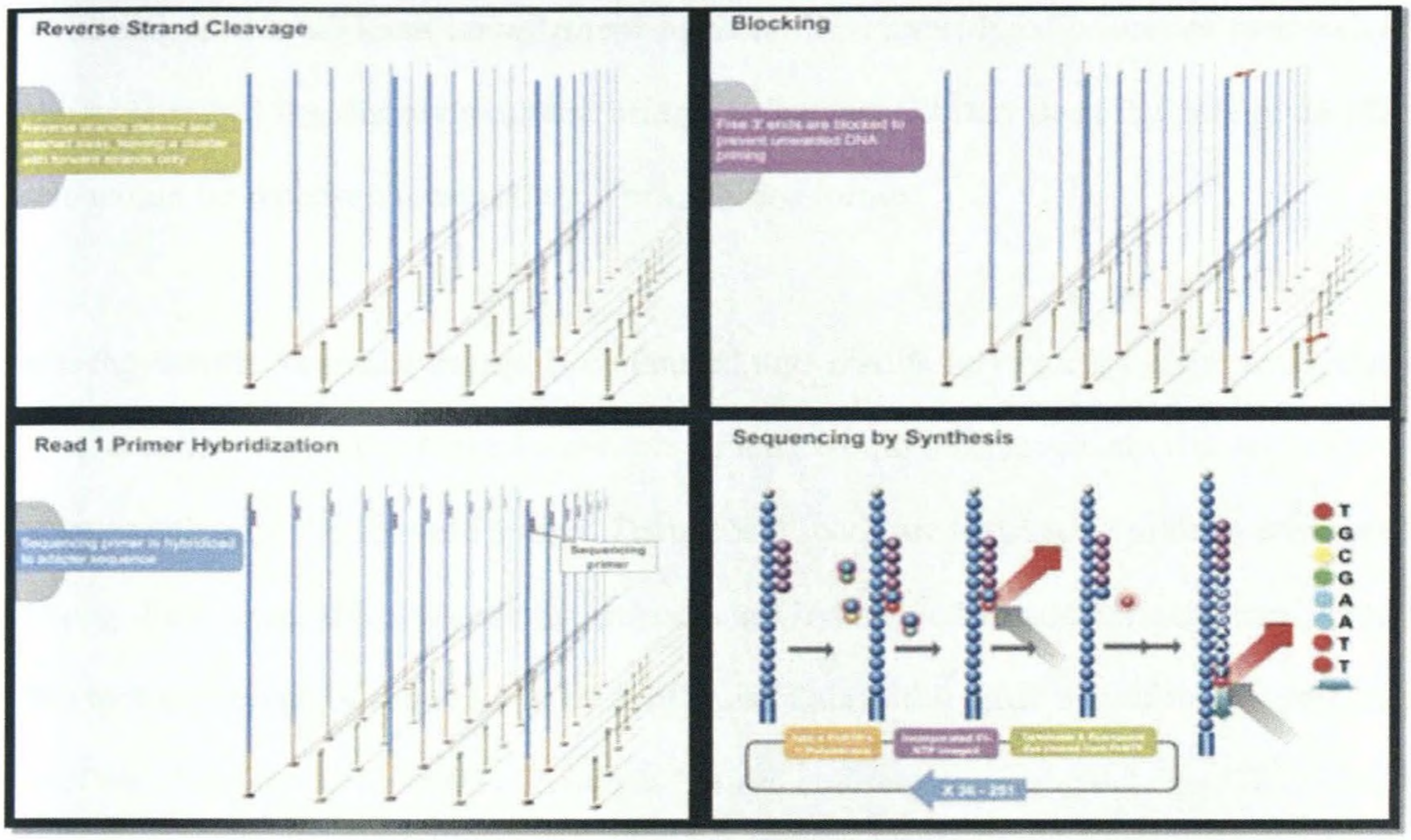


Figure 1.15 Nextera sample preparation workflow

A) Nextera transposomes with adapters
B) Tagmentation to fragment and add adaptors
C) Limited cycle PCR to add sequencing primer sequences and indices (Illumina, 2013b)

Figure 1.16 below illustrates the mechanism of cluster generation, bridge amplification & sequencing involved in Illumina MiSeq next generation sequencing platform.





(Illumina, 2013a)

Figure 1.16 Mechanism of cluster generation, bridge amplification & sequencing

The DNA insert flanked with adapters are then subjected to cluster generation followed by bridge amplification and will be sequenced by synthesis mechanism (Illumina, 2013a)

As mentioned before 'cluster generation' is the second step in the MiSeq sequencing workflow.

As illustrated in Figure 1.16, the single DNA molecule will be binding to the surface of Illumina MiSeq chip and would be amplified on the surface by getting hybridized to the primer lawn. Bound libraries then would begin extending by polymerases. It is fascinating to observe the random binding of the individual molecules to the flow cell.

Next, the double stranded DNA molecule will be denatured and the original template will be washed away. The newly synthesized strand would then get covalently attached to the flow cell surface. Following this, the single stranded molecules flip over and form a bridge by

hybridizing to the adjacent complement primers. The hybridized primer is then extended by polymerases and the double stranded bridge is formed. At this stage the bridge amplification cycle would be repeated until multiple bridges are formed.

Later the double stranded bridge is denatured and results in two copies of covalently bound single stranded templates. Next the reverse strands would be cleaved and washed away leaving a cluster with only the forward strand. Then free 3' ends are blocked to prevent unwanted DNA priming. Following this sequencing, primers are hybridized to adapter sequence. Finally this leads to the concept of sequencing by synthesis. This is the third step of the MiSeq sequencer workflow. This approach is also known as 'paired end sequencing' (Illumina, 2013a). The final step in the MiSeq workflow is to analyse the data. MiSeq reporter software (MRS) available on the MiSeq personal sequencer itself is able to align and assemble sequences to deliver results up to genus level (Illumina, 2013a).

1.7.4 Other Next Generation Sequencing platforms

The platforms for massively parallel DNA sequencing are in demand today due to its promising nature. The Roche/454 FLX, the Illumina/Solexa Genome Analyser and the Applied Biosystem SOLiD™ system were the first to be introduced to the market. In addition the Helicos Heliscope™ and Pacific Biosciences SMRT instruments (Mardis, 2008) were also brought in. Table 1.1 gives an account of these platforms.

The pace at which the changes are taking place in this field is quite rapid, with the technology being improved each year and new platforms are being introduced with significant modifications. In 2011, another three major new sequencing platforms were released: Ion Torrent's PGM, Pacific Biosciences' RS and the Illumina MiSeq (Quail et al., 2012). All such improved sequencers have the capability to generate massive amounts of informative sequencing data. However, key differences do exist between the quality of data and the applications it supports (Quail et al., 2012).

Table 1.1 Comparison of Next Generation Sequencing platforms

Platform	Library/ template preparation	NGS Chemistry	Advantages	Disadvantages	Biological Application
Roche/ 454's GS FLX Titanium	Frag, MP/ emPCR	PS	-Longer reads improves mapping in repetitive regions - Fast run times	- High reagent cost - High error rates in homo-polymer repeats	- Bacterial and insect genome de novo assemblies - Medium scale (<3 Mb) exome capture - 16S Metagenomics
Illumina/ Solexa's GA	Frag, MP/ Solid phase	RTs	-Currently the most widely used platform in the field	-Low multiplexing capability of samples	-Variant discovery by whole-genome re-sequencing or whole exome capture -Gene discovery in metagenomics
Life/APG SOLiD 3	Frag, MP/ emPCR	Cleavable probe SBL	-Two base encoding provides inherent error correction	-Long run times	-Variant discovery by whole-genome re-sequencing or whole exome capture -Gene discovery in metagenomics
Polonator G.00	MP only/ emPCR	Non-cleavable probe SBL	-Least expensive platform -Open source to adapt alternative NGS chemistries	-Users are required to maintain & quality control reagents -Shortest NGS read lengths	-Bacterial genome re-sequencing for variant discovery

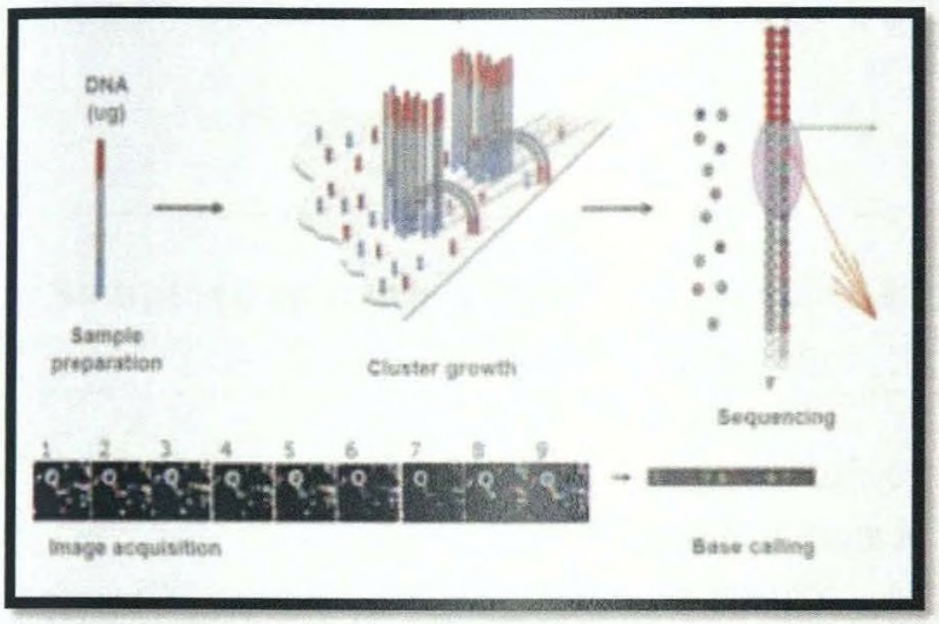
*Average read-lengths. ‡Fragment run. §Mate-pair run. Frag, fragment; GA, Genome Analyzer; GS, Genome Sequencer; MP, mate-pair; N/A, not available; emPCR, emulsion PCR; NGS, next-generation sequencing; PS, pyrosequencing; RT, reversible terminator; SBL, sequencing by ligation; SOLiD, support oligonucleotide ligation detection (Mardis, 2008)

The Ion Torrent PGM uses semiconductor technology to detect the release of protons with the incorporation of nucleotides during the DNA synthesis. DNA fragments with specific adapter sequences are linked and clonally amplified by emulsion PCR on the surface of a 3-micron diameter beads, known as Ion Sphere Particles (Quail et al., 2012). The template beads are loaded into proton sensing wells that are fabricated on a silicon wafer and sequencing is primed from the specific location in the adapter sequence. Each of the four bases is introduced sequentially (Quail et al., 2012).

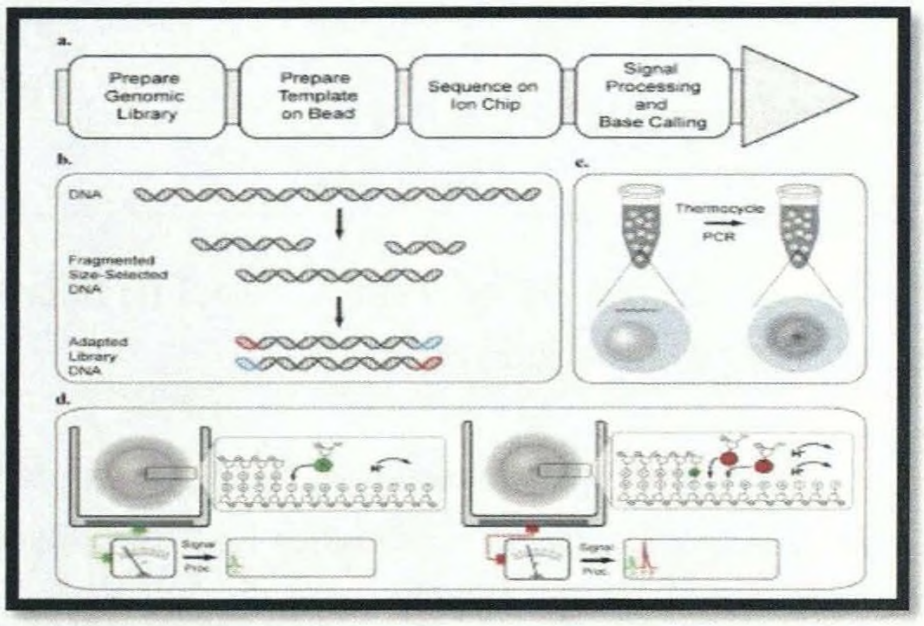
The sequencing technology is led by Illumina today with their 'sequencing by synthesis' approach, utilizing fluorescently labelled 'reversible terminator' nucleotides on clonally amplified DNA fragments restrained on an acrylamide coating on the surface of a glass flow cell (Quail et al., 2012). The Illumina Genome Analyser and more recently the HiSeq 2000 sets the standard for high throughput massively parallel sequencing. Adding to this in 2011, Illumina also released a lower throughput fast turnaround instrument; the MiSeq, directed at small scale laboratories and the field of clinical diagnostics (Quail et al., 2012).

The MiSeq records the highest throughput per run (1.6 Gb/run, 60 Mb/h) and lowest error rates. The 454 GS Junior produced the longest reads (up to 600 bases) and most contiguous assemblies but had the lowest throughput (70 Mb/run, 9 Mb/h) (Loman et al., 2012). Run in 100bp mode, the Ion Torrent PGM has the highest throughput (80–100 Mb/h) while the MiSeq has the highest accuracy (Loman et al., 2012).

Table 1.2 and Figure 1.17 give a brief comparison between Illumina MiSeq bench top genetic analyser and Ion Torrent personal genome machine. Accordingly, MiSeq uses the 'reversible terminator sequencing by synthesis' technology and Ion Torrent uses the 'semiconductor' mechanisms.



a) Illumina MiSeq



b) Ion Torrent PGM

Figure 1.17 Comparison between Illumina MiSeq and Ion Torrent PGM

Illumina MiSeq employees the 'reversible terminator sequencing by synthesis' mechanism whereas the Ion Torrent PGM uses 'semiconductor' mechanisms, picture source: (Illumina, 2013a, BGI, 2014).

Table 1.2 Comparison between Illumina MiSeq and Ion Torrent PGM

	PGM	MiSeq
Out Put	10 MB–100 MB	120 MB–1.5 GB
Read length	~200 bp	Up to 2 × 150 bp
Sequence time	2 h for 1 × 200 bp	3 h for 1 × 36 single read 27 h for 2 × 150 bp pair end read
Sample preparation time	8 samples in parallel in less than 6 h	As fast as 2h, by 15 minutes hands on time
Sequencing method	Semiconductor technology with a simple sequencing chemistry	Sequencing by synthesis (SBS)
Potential for development	Various parameters (Read length, cycle time, accuracy, etc.)	Limited factors, major concentrate in flow cell surface size, insert sizes, and how to pack cluster in tighter
Input amount	μg	ng (Nextera)
Data analysis	Off instrument	On instrument

MB, Mega bite; GB, Giga bite; bp, base pairs; h, hours; μg, micro gram; ng, nanogram

(Liu et al., 2012)

1.7.5 Pre-eclampsia studies carried out using Metagenomics NGS

There are no pre-eclampsia studies carried out using metagenomics next generation sequencing. As a result the research project discussed in this thesis remains the first study carried out to detect and identify infectious agents present in the placental tissue samples of women with pre-eclampsia using metagenomics next generation sequencing technique. Accordingly, metagenomics analysis was carried out on 55 placental tissue samples of women with pre-eclampsia and 55 normotensive women using the Illumina MiSeq Personal Genome Sequencer.

1.8 Objectives

1.8.1 General Objectives

The objective of this research project was to study the viruses, bacteria and fungi present in the placental tissues of women with preeclampsia and normotensive pregnant women using molecular genetic techniques.

1.8.2 Specific Objectives

Phase 1 of the study

- To extract the microbial DNA from the respective patient samples.
- To amplify the 16S rRNA gene of bacteria, 28S rRNA gene of fungi and the whole genome of viruses present in the placental tissue samples
- To identify the infective organism(s) responsible for the cause of pre-eclampsia using cloning based Sanger sequencing.
- To find whether there is an association between pre-eclampsia and infectious agents

Phase 2 of the study

- To carry out bacterial metagenomics next generation sequencing on the placental tissue samples obtained from the 55 women with pre-eclampsia
- To carry out bacterial metagenomics next generation sequencing on the placental tissue samples obtained from the 55 normotensive women
- To detect and identify the bacteria further in order to find the association of bacteria with pre-eclampsia.
- To compare the two latest metagenomics approaches; which is the '16S Metagenomics Next Gen Sequencing' methodology and the 'PCR Independent Metagenomics Next Gen Sequencing' methodology.

2 METHODOLOGY

2.1 Study design

This was a case control study where 55 women with pre-eclampsia and 55 normotensive pregnant women matched for age, Body Mass Index (BMI) and parity delivering via caesarean section were recruited for the study. Taking into consideration the possibility that past obstetric history as well as labour may have an effect on risk of infection; this study was confined to primiparous women with preeclampsia and to primiparous controls that underwent elective caesarean section prior to going in to labour spontaneously or induction of labour. The study was carried out in two phases; in phase 1 the samples were screened for the presence of bacteria and fungi by PCR for the 16S rRNA and 28S rRNA gene. Viral Metagenomics was performed for the identification of viruses. In phase 2, the samples which were positive for the 16S rRNA gene were subjected by next generation sequencing on an Illumina MiSeq platform.

2.2 Ethical considerations

Ethical clearance was obtained for this study from the Ethics Review Committee of the Faculty of Medicine, University of Colombo and the research project conformed to the provisions of the Declaration of Helsinki. At all times privacy and confidentiality of the participants was maintained. Written informed consent was attained from the participants enabling them to make the decision personally without any influence. The data collection booklet was designed to ensure confidentiality of the information gathered. Both participants were given a code (subject study number) and the personal details of the participants were kept separate from the data sheet under lock and key. The data base and the computer containing the data base was password protected.

This study was carried out to ascertain whether there was an association between infectious agents with pre-eclampsia. The findings of this study would benefit the subjects and also other pregnant women in the future. Volunteer participants were given the liberty to question the investigators about the tests, procedures and other required information and were given the contact numbers of the investigators in case they needed to clarify any doubts about the study. As the study was open to all ethnic groups in Sri Lanka, consent forms and information sheets were printed and were made available in Sinhala, Tamil and English so that the participants could choose the language of their choice.

All volunteers were literate enough to read and understand the information sheet and sign the consent form. If the participants had any questions about the study they were free to ask the investigator and were answered before consenting. Any woman was free to withdraw her consent at any time, with no penalty or effect on medical care or loss of benefit received from the hospital. This study has a social and scientific validity as it would help to understand the role of infectious agents in association with preeclampsia. This will be helpful for improved clinical management of patients with preeclampsia. The study used more advanced molecular genetic techniques with higher accuracy to ensure the scientific validity.

2.3 Definition of Pre-eclampsia

The International Society for the Study of Hypertension in Pregnancy (ISSHP) defines pre-eclampsia as *de novo* hypertension (systolic blood pressure (SBP) of ≥ 140 mmHg and diastolic blood pressure (DBP) of ≥ 90mmHg) subsequent to 20 weeks of gestation in an earlier normotensive pregnant woman, which proceeds back to normal by the end of the third month postpartum, in association with proteinuria of ≥ 300 mg protein/day on a 24 hour urine collection (Brown et al., 2001).

In the recruitment hospitals where this study was conducted, the 'Urine Protein Heat Coagulation Test' (HCT) was performed by the nurse or the midwives. Dissanayake et al had validated the detection of proteinuria using the HCT, and confirmed a cut off of ≥ 1+ as being equivalent to >300mg protein/day (Dissanayake et al., 2004). As such pre-eclampsia for the purpose of this study was defined as *de novo* hypertension (systolic blood pressure (SBP) of ≥ 140 mmHg and diastolic blood pressure (DBP) of ≥ 90mmHg) subsequent to 20 weeks of gestation in an earlier normotensive pregnant woman, which proceeds back to normal by the end of the third month postpartum, in association with ≥ 1+ proteinuria detected through the urine protein heat coagulation test (HCT).

2.4 Recruitment of subjects

2.4.1 Recruitment of women with pre-eclampsia (cases)

This study was conducted in primiparous (P_1C_0) women with no known risk factors for pre-eclampsia. These patients were recruited from the De Soysa Hospital for Women and the Castle Street Hospital for Women, Colombo and also from Kethumathi Hospital, Panadura during the period January 2010 to June 2011. The protocols used for the patient management in the recruiting hospitals were similar in terms of access to drugs, investigations and other facilities. Written informed consent was obtained from all participants.

Strict inclusion and exclusion criteria were considered before recruiting the participants to the study. Medical data of patients entered in the antenatal clinic card, admission card and in patient records were extracted and considered before recruiting the patient to the study. All volunteers recruited for the study were questioned personally to obtain past medical data. Inclusion criteria for the selection of cases were women with pre-eclampsia (women with new onset hypertension of systolic and diastolic blood pressure of $\geq$ 140/90mmHg and Proteinuria of $\geq$ 1+ on HCT) undergoing delivery through caesarean section.

Vaginal delivery, forceps and vacuum assisted vaginal delivery, ischemic heart disease, cerebrovascular accidents, insulin or non-insulin dependent diabetes mellitus, Body Mass Index $\geq$ 30 kg/m^2 based on height and weight measured at the antenatal booking visit or post-partum in case of in booked pregnancies, multiple gestation, gestational diabetes in the current pregnancy, connective tissue disorder, long term steroid uses, endocrine disorders and valvular heart disease were the exclusion criteria for recruitment of patients to the study. In addition,

obstetric conditions that confer an increased risk of infection (i.e. premature rupture of membranes, preterm labour and women with any known infection) were also considered as exclusion criteria.

Severe maternal morbidity was defined as of: systolic blood pressure (SBP $\geq$ 160mmHg); diastolic blood pressure (DBP $\geq$110 mmHg); proteinuria $\geq$ 3+ on HCT (> 150 mg/dL); thrombocytopenia (< 100x 10^9/ L); impaired liver function (Aspartate aminotransferase (AST) and Alanine aminotrasnferase (ALT) $\geq$ 70 U/L); renal failure requiring dialysis; placental abruption and eclampsia (Dissanayake et al., 2007). Early onset preeclampsia was defined as the disease onset at < 34 weeks of gestation. All these clinical features are associated with maternal morbidity and mortality. Severe prenatal morbidity was defined as: delivery at < 34 weeks of gestation and birth weight < 2kg (Dissanayake et al., 2007, Dissanayake et al., 2004).

2.4.2 Recruitment of normotensive pregnant women (controls)

Recruitment of normotensive pregnant women was carried out simultaneously with the recruitment of women with pre-eclampsia from January 2010 to June 2011. The normotensive women were matched for parity, age and Body Mass Index (BMI) from the same recruiting hospitals as above (De Soysa Hospital for Women, the Castle Street Hospital for Women, Colombo and the Kethumathi Hospital, Panadura).

Normotensive pregnant women undergoing delivery through caesarean section due to medically uncomplicated reasons were referred to the study. Similar to the exclusion criteria of selecting cases; vaginal delivery, forceps and vacuum assisted vaginal delivery, ischemic

heart disease, cerebrovascular accidents, insulin or non-insulin dependent diabetes mellitus, Body Mass Index $\geq$ 30 kg/m^2 based on height and weight measured at the antenatal booking visit or post-partum in case of in booked pregnancies, multiple gestation, gestational diabetes in the current pregnancy, connective tissue disorder, long term steroid uses, endocrine disorders and valvular heart disease were the exclusion criteria for the recruitment of controls to the study. In addition normotensive pregnant women with pregnancy complications, intra uterine growth restrictions, pre-term delivery (that is before 37 weeks and having a baby with low birth weight of < 2kg), premature rupture of membranes and preterm labour were also excluded from the study. If this initial inclusion and exclusion criteria were met then they were screened for parity, age and BMI to ensure that the women in the control group matched the women with pre-eclampsia recruited to the study.

Accordingly, 55 normotensive non proteinuric pregnant women matched for parity, age and BMI were selected as controls. Women with pre-eclampsia and normotensive pregnant women recruited to the study were compared on a daily basis to ensure recruitment of proper matched cases and controls. Only women undergoing caesarean section were selected while recruiting women with pre-eclampsia (cases) and normotensive pregnant women (controls). All voluntary participants (cases and controls) recruitment data were entered in to a database for statistical analysis maintaining the confidentiality and privacy of the subjects.

2.4.3 Comparing two cohort studies

A similar study was carried out in the period of 2001- 2003 by Dissanayake et al, where 180 nulliparous women with pre-eclampsia and 180 nulliparous normotensive pregnant women were recruited for a study into genetics of pre-eclampsia (Dissanayake et al., 2007). Same inclusion and exclusion criteria were followed in both the studies but the present study was limited to women with pre-eclampsia who delivered only through caesarean section.

The study carried out in 2001-2003 recruited nulliparous women (women who have never given birth and were in their first pregnancy) and similarly the current study was also carried out on primiparous women (women who were in their first pregnancy). The study by Dissanayake et al was carried out at De Soysa Maternity Hospital, Colombo and Castle Street Hospital for Women, Colombo. The current study was carried out at the De Soysa Maternity Hospital, Castle Street Hospital for Women, Colombo and Kethumathi Maternity Hospital in Panadura. All hospitals at which these women were recruited are government hospitals following the same standard procedures in diagnosing and managing women with pre-eclampsia.

These two cohorts (women with pre-eclampsia as case group and normotensive pregnant women as control group) recruited maintaining same inclusion and exclusion criteria from similar hospitals is analysed and compared in this thesis.

2.5 Sample size calculation

Sample size was calculated according to a previous research study that reported on the association of infectious agents found in placental tissues obtained from women with pre-eclampsia and normal pregnant women (Barak, 2007). Based on this report the sample size was calculated using the 'sample size calculation for matched case control studies' function of the "Stats Direct" statistical software package. Given that the probability of exposure of controls was 0.15 and the increased risk associated with the presence of infectious agents in the placenta (odds ratio) was 3.5, 55 cases and 55 matched controls would be required to have 80% probability of detecting a real effect and 5% probability of accepting the null hypothesis by chance.

2.6 Sample collection

The following samples were collected from both the women with preeclampsia and normotensive pregnant women recruited to the study. Stringent aseptic measures were followed throughout the collection and analysis of the samples to prevent contamination.

i. Placental tissue

After the delivery of the baby the placenta was delivered and placed on a sterile tray. A small piece of the placental tissue (cotyledon close to the insertion of the umbilicus) was immediately dissected by the surgeon and was placed in a sterile insulated container on ice. This was immediately transferred to the laboratory and was stored in the -80°C freezer.

ii. Amniotic Fluid

Approximately 5-10ml of amniotic fluid was collected into a sterile container at the time of making the caesarean section incision.

iii. Blood

3ml of venous blood was collected into a 5ml heparin tube at the time of cannulation for the caesarean section.

iv. Urine

10ml of urine was collected on to sterile containers at the time of catheterization for the caesarean section.

2.7 Sample transportation and storage

Dissected placental tissue sample, amniotic fluid, urine and blood samples collected at the time of delivery were immediately transported within 15- 30 minutes to the Human Genetics Unit at the Faculty of Medicine, University of Colombo in an insulated container with ice. They were stored in the -80°C freezer in a labelled sterile container.

PHASE 1

2.8 Detection of bacteria from the placental tissue samples

2.8.1 DNA extraction

Phase 1 began with the extraction of DNA from the placental tissue samples obtained from women with pre-eclampsia and normotensive pregnant women using QIAamp® DNA MINI kit (QIAGEN Ltd, USA). This kit provided a fast and easy method for purification of total DNA (e.g., genomic, viral, mitochondrial) from whole blood, plasma, serum, buffy coat, bone marrow, other body fluids, lymphocytes, cultured cells, tissues and forensic specimens (QIAGEN, 2010). 25mg of the placental tissue was excised into small pieces with the use of a sterile blade and transferred into a sterile 1.5ml micro-centrifuge tube (Usually 1mg of tissue would yield approximately 0.2-1.2µg of DNA). 180µl of Tissue Lysis buffer (ATL) was added for complete lysis of the tissue. 20µl of proteinase K was added, mixed by vortexing and incubated in a shaking water bath at 56°C till the tissue was completely lysed. Micro-centrifuge tubes were briefly centrifuged to remove drops from the inside of the lid. 200µl Cell Lysis buffer (AL) was added and mixed by pulse vortexing for 15seconds and incubated at 70°C for 10 min. To this sample 200µl of 96-100% ethanol was added and contents mixed by pulse vortexing.

This mixture was carefully transferred to a QIAamp Mini spin column without wetting the rim and centrifuged at 8000rpm for 1 min. The filtrate was discarded and the mini spin column placed in a fresh collection tube. To this, 500µl Wash buffer (AW1) was added and centrifuged for 8000rpm for 1 min. Subsequently, 500µl of buffer AW2 was added and centrifuged at 14,000 rpm for 3 min. Finally, the spin columns were placed in a sterile 1.5 ml micro-centrifuge tube and 200µl buffer AE (10mM Tris.Cl; 0.5mM EDTA; pH 9.0) added. After incubating for

5 minutes it was centrifuged at 8000 rpm for 1 min and the purified DNA stored at -20°C. The expected yield from 25mg of tissue would be approximately 10-30µg of DNA (QIAGEN, 2010).

2.8.2 Polymerase Chain Reaction (PCR)

For the detection of bacteria, 16S rRNA gene of bacteria was amplified using the 'Polymerase Chain Reaction' (PCR) method. The polymerase chain reaction amplifies a single or a few copies of a fragment of DNA into thousands and millions of copies. The method comprises of repeated heating cooling cycles carried out on a thermo-cycler and was a method initiated by Dr. Kary Mulis in 1983 (Lynch and Brown, 1990, Saiki et al., 1988, Saiki et al., 1985, Coates et al., 1991). Amplifying the 16S rRNA gene and analysing its sequence is the basis for bacterial identification. This method used a set of universal primers 16S rRNA BSF8/20 (5'-AGA GTT TGA TCC TGG CTC AG -3') and 16S rRNA BSR514/18 (5'- ATT ACC GCG GCCT GCT GGC -3') to amplify the 16S rRNA gene of bacteria. This will result in a 511 bp band when run on a 1.5% Agarose gel.

Thus, for 5µl of the 5x PCR reaction buffer (10mM Tris-HCl, 50mM KCl, 1.5mM $MgCl_2$, pH 8.5, 20°C) 1.5µl of 25mM $MgCl_2$, 1.25µl of 2mM dNTP' s (dATP, dGTP, dTTP and dCTP) was added. 0.3µl of each 50µM forward and reverse 16s rRNA primers (15pmol) and 0.2µl (1U) of Go Taq DNA polymerase (Promega, USA) was added to the above mixture along with 3µl of purified DNA. Volume of the PCR mixture was adjusted to 25µl using distilled water (dH_2O).

The previously named reaction mixtures were placed inside the ABI 2720 thermo-cycler (Applied Bio-Systems, Life Technologies USA). Initial denaturation of the genome was set at 95°C for 5 minutes, then 40 cycles of repeated heat denaturation of 30s at 94°C, annealing of the primers at 61.5°C for 30seconsd with the extention taking place at 72°C for 30 s. This reaction was concluded with a final extention at 72°C for 10 minutes and was held at 4°C. The presence of bacteria was confirmed by the presence of the amplified 16S rRNA gene detected by agarose gel electrophoresis.

2.8.3 Agarose gel electrophoresis

This is the standard technique used to separate and detect the amplified nucleic acid product (Barril, 2012, Sambrook, 2001). Gel tray was sealed from either sides using tape to make a mould.

0.50g of molecular biology grade 'Agarose' (Helena BioSciences, UK) was measured using a digital weighing scale and was added to a conical flask. To make a 2% gel 25ml of 1x Tris Borate Ethylene di amine tetra acetic acid buffer (TBE) was added to the agarose powder. Agarose was dissolved in TBE buffer by heating it for 1 min in a microwave and 0.5µg/ml of Ethidium bromide was added. The hot gel mixture was then poured into the casting tray with a comb of the precise number of wells and kept aside to set into a gel. Once the gel was set, combs were removed and the gel placed inside the gel electrophoresis apparatus with 1x TBE buffer. The gel was placed 0.5-1mm above the casting tray to make sure the well was formed properly (Sambrook, 2001).

8µl of the PCR product mixed with 2µl of 6x loading dye (15% W/v Ficoll, 0.255 (w/v) bromophenolblue, and 0.25% (w/v) xylene cyanol) and loaded onto the gel. 1µl of 6x loading dye mixed with 1µl of the marker DNA (ladder) was loaded onto the first well of the gel. DNA markers are available in different sizes and could be purchased commercially as per the need. A 100bp ladder (Promega, USA) was used as the expected amplified product size was approximately 500bp. A positive and a negative control samples were also run along with the experimental samples.

After loading the gel, a voltage of 60V (1-8V/cm) was applied. When the voltage was applied the negatively charged DNA molecules migrated towards the anode. Smaller fragments of DNA moved faster compared to the larger fragments and this enabled a size separation of the DNA molecule. Depending on the concentration of the gel (Gel percentage) and the amount of voltage applied the DNA migration rate and resolution would vary. After 30 minutes, the gel was observed under an UV trans-illuminator. The added Ethidium bromide intercalated with the DNA particles and fluorescence was detected under UV light. This aids in visualizing the separated DNA fragments as per their sizes (Dissanayake, 2004). Accordingly a 500bp band was detected confirming the amplification of 16S rRNA gene which indicated the presence of bacteria. Gel was pictured by the gel documentation system and was saved for future analysis.

2.8.4 Automated sequencing

Exo- SAP purification was carried out by adding 1µl of 10x buffer Exo 1, 1µl of 10x buffer SAP, 1µl of the SAP enzyme and 0.25µl of Exo1 enzyme (Fermentas, Life Sciences, USA) to the 5µl of PCR product. dH_20 was added to make the volume to 10µl and the enzymatic reaction was allowed to take place at 37°C for 15 minutes in the thermo cycler (2720 Applied bio-system, USA). This reaction was inactivated at 80°C for 15 minutes and was held at 4°C before commencing the cycle sequencing reaction.

Cycle sequencing mixture was prepared by adding 2µl of 5x buffer, 1µl of the 12.5µM 16S BSF primers and 0.8µl of cycle sequencing mix supplied by the Big Dye® termination v3.1 cycle sequencing kit (Applied bio-systems, USA). 3µl of the Exo-SAP purified PCR product was added and the reaction volume was adjusted to 10µl with dH_2O. This reaction was carried out on ice in a dark laboratory setting as the cycle sequencing mix is light sensitive. The prepared reaction was subjected to initial denaturing at 96°C for 1 minute followed by 25 cycles of denaturing at 96°C for 30 seconds, annealing temperature at 50°C for 15 seconds and extension at 60°C for 4 minutes. Cycle sequencing was completed with a final extension at 28°C for 1 minute and was held at 4°C in the thermo cycler.

Next step was to clean the cycle sequencing reaction with the help of Just-a-Plate™ sequencing reaction clean up kit for Big Dye® Terminator kit (AZCO Biotech, Inc.). Accordingly, the cycle sequencing product obtained was thoroughly mixed with 2µl of binding buffer supplied by the sequencing reaction clean up kit above and 25.5µl of 100% ethanol. This was subjected to centrifugation at 13000 rpm for 20 minutes at 4°C in a micro-centrifuge. Contents were pipetted out without touching the wall of the tube and were kept to air dry in dark conditions. The dried

tubes were washed with 40µl of 70% ethanol and were placed on a dry block set at 65°C for 5 minutes. The purified sequencing products were eluted in 10.3µl of Hi- Di™ formamide (Applied Bio-systems Life Technologies, USA). The contents were centrifuged at 13000 rpm for 15 minutes at 4°C, denatured at 95°C for 5 min and were placed in -20°C prior to automated sequencing. All steps were carried out in dark conditions.

2.8.5 Analysis of 'Electrophorogram'

After the completion of the automated sequencing process, the next step was to analyse the resulting electrophorogram. The sequence was analysed with the help of Bio Edit 7.5.0.3 software and was copied into a FASTA format. FASTA sequence obtained was BLAST against the microbial genome database available at the NCBI website. This facilitated the identification of the bacteria present in the sample. However, if the electrophorogram appears distorted 'multiple overlapping nucleotide peaks in the electrophorogram showing distorted sequence', such an electrophorogram can only be due to the presence of different sequences generated from multiple organisms. In such a situation the PCR amplification of the 16S rRNA gene was cloned and sequenced.

2.8.6 Cloning Experiment

2.8.6.1 Gel purification of the PCR product

First step prior to cloning is the gel purification of the PCR product. Accordingly, all samples which gave positive results were subjected to a repeat PCR to amplify the 16S rRNA genes common to all bacteria. PCR amplified products were then gel purified using Wizard® SV Gel and PCR Clean-Up System (Promega, USA). 50µl of PCR amplification reaction was loaded

on to a 1% gel and was allowed to run on a gel electrophoresis apparatus. Once the desired band which is 500bp, was properly separated it was excised from the gel and was placed in a 1.5 ml micro centrifuge tube.UV protecting glasses along with the facemask was worn while excising the band from the gel. Exposure of Ultra violet light ought to be curtailed as far as possible in order to reduce the formation of pyrimidine dimers.

10µl of membrane binding solution supplied with the Wizard® SV Gel and PCR Clean-Up System (Promega, USA) was added to per 10mg of gel slice. It was vortexed and incubated at 60°C until the gel slice was completely dissolved. The dissolved gel mixture was transferred on to the SV mini column and incubated at room temperature for 1 minute. It was centrifuged at 14,000 rpm for 1 minute and the flow through was discarded. 700µl of membrane wash solution was added and centrifuged at 14000 rpm for 1 minute. This washing step was repeated with 500µl of membrane wash solution which has pre-added 95% ethanol. After centrifuging for 5 minutes at 14000 rpm the mini column was placed at room temperature for 5 minutes to evaporate any remaining ethanol. Finally, purified DNA was eluted in 50µl of nuclease free water by centrifugation at 14000 rpm for 1 minute. Eluted purified DNA was stored in a -20°C freezer till the commencement of cloning experiments.

2.8.6.2 Cloning the target DNA using pGEM- T® vector

Gel purified DNA product was cloned using pGEM- T® vector in accordance with the user manual (Promega, 2010) and the procedure followed is described in this section. The pGEM-T® vector and control insert DNA tubes were briefly centrifuged to collect the contents at the bottom of the tubes. 2x ligation buffer were vortexed vigorously before each use. The

experiment was carried out in 0.5ml tubes known to have low DNA-binding capacity. Ligation reactions were set up as illustrated in Table 2.1 below;

Table 2.1 Ligation reaction

Reaction Component	Standard reaction (µl)	Positive control (µl)	Background control (µl)
2X Rapid Ligation Buffer, T4 DNA Ligase	5	5	5
pGEM®- T vector (50ng)	1	1	1
PCR product	3*	-	-
Control Insert DNA	-	2	-
T4 DNA Ligase (3 Weiss units/µl)	1	1	1
Nuclease free water to a final volume of	10	10	10

The prepared reaction mixture was mixed by pipetting and was incubated at room temperature for 1 hour followed by incubation at 4°C overnight. Only T4 DNA ligase supplied with the kit was used to avoid exonuclease activity that may remove the terminal deoxy thymidines and may degrade the T over hangs on the vector. Since 2x Rapid Ligation Buffer contained ATP, which degrades during temperature fluctuations, single use aliquots of the buffer were prepared prior to the commencement of the ligation procedure. Ligation reactions using this buffer were incubated overnight to produce the maximum number of transformants and increase the number of colonies after transformation (Promega, 2010).

2.8.6.3 Transforming the recombined pGEM®-T vector into competent cells JM109

Next step following ligation was the transformation of recombinant vector into the competent cells. High efficiency JM109 competent cells ($\geq 1x10^8$cfu/µg DNA) were used for the transformation reaction. These cells were provided with the pGEM®-T vector kit II (Promega, USA). The genotype of JM109 is *rec*A1, *end*A1, *gyr*A96, *thi, hsd*R17 (rK-,mK+), *rel*A1, *sup*E44, Δ(*lac-pro*AB), [F, *tra*D36, *pro*AB, *lac*I^{q}ZΔM15] (Messing et al., 1981, Promega, 2010).

Before the transforming experiment commenced plates with ampicillin/IPTG/X-Gal were prepared with the commercially available imMedia™ Amp Blue (Invitrogen™, Life Technologies, USA). Prepared plates were equilibrated to room temperature. Tubes containing the ligation reactions were centrifuged to collect the contents at the bottom. 2µl each of ligation reaction was added to a sterile 1.5ml micro centrifuge tube on ice. Another tube with 0.1ng uncut plasmid were prepared and kept on ice for the determination of the transformation efficiency of the competent cells. JM109 High efficiency competent cells were placed in an ice bath until just thawed. The cells were mixed by gently flicking the tubes and avoiding excessive pipetting since the competent cells are extremely fragile.

50µl of cells were then transferred into each prepared tube and 100µl competent cells added to the tube containing the 0.1ng uncut plasmid. The tubes were gently flicked to mix and were placed on ice for 20 minutes. Cells were heat shocked for 45-50 seconds in a water bath exactly at 42°C without shaking and immediately replaced on ice for 2 minutes. 950µl of room temperature 'Super Optimal broth with Catabolite repression' medium (S.O.C medium)

(Invitrogen™, Life Technologies, USA) was added to the tubes containing cells transformed with ligation reactions and 900µl to the tube containing cells transformed with uncut plasmid.

Tubes were incubated at 37°C for 1.5 hours, shaking approximately at 150 rpm. 100µl of the transformed cultures were cultured on to duplicate LB/ampicillin/IPTG/X-Gal plates. Transformed control was plated in a 1:10 dilution on S.O.C. Medium. Plates were incubated at 37°C overnight to obtain blue and white colonies. White colonies contained the insert of interest and would appear approximately 1mm in diameter (Promega, 2010).

2.8.6.4 Transformation efficiency calculation

After 100µl of competent cells were transformed with 0.1ng of uncut plasmid DNA, the transformation reaction was added to 900µl of S.O.C. medium (0.1ngDNA/ml). From that volume, a 1:10 dilution with S.O.C. medium (0.01ngDNA/ml) was made and 100µl was plate onto two plates (0.001ngDNA/100µl). The transformation efficiency was calculated for the JM109 competent cells as follows;

$$\text{Transformation efficiency} = \frac{\text{Number of colony forming units}}{0.001\text{ng}}$$

2.8.6.5 Sub culturing

After carefully selecting the white colonies with the insert it was necessary to subculture the clones for further analysis and for the preparation of plasmids. Luria-Bertani broth (LB) was prepared by dissolving 2.5g of LB powder (SIGMA-ALDRICH, USA) in 100 ml of dH_2O. Ampicillin powder (SIGMA-ALDRICH, USA) was added to make a concentration of 125µg/ml. Approximately around 10 selected white colonies were numbered and were first touched on to LB/X-Gal/IPTG/amp plate and then sub-cultured on to tube containing 600µl LB broth/ampicillin. LB broth provided the nutrition medium needed for bacterial growth. Since the plasmid vector pGEM®-T contains Amp^r gene (Ampicillin resistant gene), the recombined bacteria would be resistant to Ampicillin. Therefore the Ampicillin added to LB broth would allow only the bacteria of interest to grow and prevent the growth of other. The sub-cultured plate and the tubes were incubated overnight at 37°C in a shaking incubator set up to shake horizontally at 150 rpm. After overnight incubation the clear LB broth with ampicillin had a misty appearance indicating the growth of the recombined bacteria containing the target insert. Sub-cultured plate was stored for future analysis.

2.8.6.6 Isolation of 'Plasmid DNA' from the recombined bacteria

Plasmids DNA from the sub cultured recombined bacterial cells were isolated using Pure Yield ™ Plasmid Mini-prep System (Promega, USA). This kit provided a rapid method to purify plasmid DNA using silica membrane columns (Promega, 2009). The kit contained endo-toxin removal washing solution which removed impurities such as proteins, RNA and endo-toxin contaminants and allowed the isolation of purified highly concentrated plasmid DNA (Promega, 2009).

Neutralization solution (NSC) was stored at 4-8°C and the rest of the kit content was stored at room temperature. The Cell Lysis Buffer (CLC) was incubated at 35°C for 30 minutes and was mixed by inverting before use. The Column wash solution was diluted with 95% ethanol in accordance to user instructions in the kit manual. 100µl of the CLC which is blue in colour was added to the 600µl of sub-cultured cells and was mixed by inverting the tubes 6 times. 350µl of cold NSC was added mixed thoroughly by inverting. This turned the mixture from blue to yellow colour.

The reaction mixture was centrifuged at maximum speed at 14000 rpm in a micro-centrifuge for 3 minutes and the supernatant (~900µl) was transferred to a Pure Yield™ mini-column without disturbing the cell pellet. The mini-column was placed in a collection tube and centrifuged at 14000rpm for 15 seconds. The flow-through was discarded and the mini-column placed in the same collection tube. To this, 200µl of endo-toxin removal wash (ERB) was added and centrifuged at a 14000 rpm speed for 15 seconds. 400µl of column wash solution was added and centrifuged at 14000 rpm speed for 30 seconds. Finally, the mini-column was placed in a sterile 1.5ml micro-centrifuge tube and 30µl of Elution buffer added to elude the plasmid DNA out. Eluted plasmid DNA was stored in -20°C freezer until it was used for further analysis.

2.8.6.7 Colony PCR

Colony PCR was carried out to ensure the isolated plasmids contained the target insert of interest by amplifying a portion of the insert. Colony PCR was performed with both insert specific primers and vector specific primers to screen for the recombinant plasmids. The insert specific universal primers 16S rRNA BSF8/20 (5'- AGA GTT TGA TCC TGG CTC AG -3') and 16S rRNA BSF514/18 (5'- ATT ACC GCG GCCT GCT GGC -3') was used to amplify the inserted DNA fragment. This resulted in a 511bp band when run on a 1.5% Agarose gel indicating a successful isolation of the plasmid DNA with the target DNA insert.

Similarly vector specific primers, T7 sequencing primer (5'- TAA-TAC-GAC-TCA-CTA-TAG-GG-3') and Sp6 sequencing primer (5'- ATT-TAG-GTG-ACA-CTA-TAG-3') were used to amplify a portion of the plasmid to ensure that the insert was present. Thus, for 5µl of the 5x PCR reaction buffer (10mM Tris-HCl, 50mM KCl, 1.5mM $MgCl_2$, pH 8.5, 20°C) 1.5µl of 25mM $MgCl^2$, 1.25µl 2mM dNTP' s (dATP, dGTP, dTTP and dCTP) was added. 0.6µl of each 25µM T7 forward primer and SP6 reverse primer (15pmol) and 0.5µl (1U) of Go Taq DNA polymerase (Promega, USA) was added to the above mixture along with 2µl of purified plasmid DNA. Volume of the PCR mix was adjusted to 50µl with dH_2O.

The above reaction mixtures were placed on the ABI 2720 thermo-cycler (Applied Bio-Systems, Life Technologies USA). Initial denaturation of the genome was set at 94°C for 2 minutes, then 35 cycles of repeated heat denaturation of 30s at 94°C, annealing of the primers at 55°C for 1 minute with extension taking place at 72°C for 2.5 minutes. This reaction was concluded with a final extension at 72°C for 10 minutes and was held at 4°C. The presence of bacteria was confirmed by the presence of the amplified inserted DNA fragment (which is the

16S rRNA gene) detected by running on a 1% Agarose gel which results in two bands; one band at 500bp mark and the other band at 650bp mark (primer and the insert).

2.8.6.8 BstZI restriction digestion

BstZ1 restriction enzyme digestion was performed for the plasmids prior to sequencing to verifying whether the insert was present in the plasmid purified. The BstZ1 restriction enzyme digestion was carried out by adding 0.2µl of 10µg/µl of Acetylated BSA to 2µl of 10X RE buffer. To this mixture 1µl of 1µg/µl concentrated plasmid DNA and 0.5µl of 10u/µl BstZ1 restriction enzyme was added. 16.3µl of dH_2O was added to make up the final volume to 20µl and mixed gently by pipetting. This reaction mixture was briefly centrifuged in a micro-centrifuge and incubated at 50°C for 3 hours. The reaction was inactivated at 65°C for 15 minutes. The reaction products were then examined by running on an agarose gel which would give a band approximately at 500-600bp indicating the presence of the insert. Uncut plasmids were used as the experimental control.

2.8.6.9 Automated sequencing of plasmids

Sequencing of plasmids was carried out using automated sequencing. In summary, the 10 white colonies carefully selected from the blue/white screening were sub-cultured and plasmid DNA isolated. The plasmid DNA confirmed for the presence of the insert DNA was subjected to automated sequencing. Accordingly, 5-10 plasmid DNA detected positive for the presence of bacteria were sequenced. The FASTA sequence of the resulting sequences was BLAST against the NCBI genbank.

2.9 Detection of fungi from the placental tissue samples

2.9.1 Amplification of the 28S rRNA gene

DNA was extracted from the placental tissue samples using the QIAamp DNA extraction kit (QIAGEN, USA). NL1 (5' GCA TAT CAA TAA GCG GAG GAA AAG-3') and NL4 (5'-GGT CCG TGT TTC AAG ACG G-3') were used as the forward and reverse universal fungal primer pair to amplify the 28S rRNA gene of fungi (Voigt et al., 1999, Chen et al., 2003, Rosen and Lim, 2012, Hurley, 2007, Yunianto, 2012). 1.5µl of 25mM $MgCl^2$, 1.25µl of 2mM dNTP's (dATP, dGTP, dTTP and dCTP) was added to 5 µl of the 5x PCR reaction buffer (10mM Tris-HCl, 50mM KCl, 1.5mM $MgCl_2$, pH 8.5, 20°C). 0.3µl of each 50µM forward and reverse NL1 and NL4 primers (15pmol) were added to the mixture along with 0.2µl (1U) of Go Taq DNA polymerase (Promega, USA). Finally 3µl of purified DNA was added and the volume of the PCR mix was adjusted to 25µl with the help of dH_2O. The above reaction mixtures were placed inside the ABI 2720 thermo-cycler (Applied Bio-Systems, Life Technologies USA). Initial denaturation of the genome was set at 95°C for 5 minutes, then 40 cycles of repeated heat denaturation of 30s at 94°C, annealing of the primers at 61.5°C for 30s with the extention taking place at 72°C for 30s. This reaction was concluded with a final extention at 72°C for 10 minutes and was held at 4°C. The presence of fungi was confirmed by the presence of the amplified 28S rRNA gene detected by gel electrophoresis.

2.9.2 Gel electrophoresis to detect the 28S rRNA gene

The product obtained from the 28S rRNA gene amplification was subjected to gel electrophoresis using a 1.5% Agarose gel. This gel was made using 0.38g of agarose dissolved with 25ml of 1X TBE buffer topped up with 5µl of ethidium bromide. Once the gel was set at room temperature it was dipped in 1X TBE buffer and 8µl from the PCR product was loaded

with 2µl of loading dye along with a 100bp DNA ladder. Gel was then subjected to a voltage of 65V for 30 minutes in a gel electrophoresis apparatus. When visualized under UV light a band at 550bp indicated the presence of 28S rRNA gene. This signified the presence of fungi in the placental tissue sample. The samples resulting in a band at 550bp was considered positive for the presence of fungi and the samples where the band was absent were considered negative.

2.9.3 Automated sequencing and analysis of electrophorogram

The PCR product was purified with the treatment of Exo-SAP cleanup process. Exonuclease 1 (Exo1) was used to remove the excess primers in the PCR mixture and Shrimp Alkaline Phosphatase (SAP) was used to remove the additional deoxy nucleotide tri phosphate (dNTPs). 5µl of PCR product was mixed with 1µl of 10x buffer Exo 1, 1µl of 10x buffer SAP, 1µl of the SAP enzyme and 0.25µl of Exo1 enzyme (Fermentas, Life Sciences, USA). dH_20 was added to make the volume to 10µl and the enzymatic reaction was allowed to take placed at 37°C for 15 minutes in the thermo cycler (2720 Applied bio-system, USA). This reaction was inactivated at 80°C for 15 minutes and was held at 4°C before commencing the cycle sequencing reaction.

Cycle sequencing was carried out with the ABI PRISM Big Dye® terminator v3.1 cycle sequencing kit (Applied Biosystems, USA). Accordingly cycle sequencing mixture was prepared by adding 2µl of 5x buffer, 1µl of the 12.5µM NL1 forward primer and 0.8µl of cycle sequencing mix supplied by the Big Dye® termination v3.1 cycle sequencing kit (Applied bio-systems, USA). 3µl of the Exo-SAP purified PCR product was added and the reaction volume was adjusted to 10µl with dH_2O. This reaction was carried out on ice in a dark laboratory setting as the cycle sequencing mix was light sensitive. The prepared reaction was subjected to initial denaturing at 96°C for 1 minute followed by 25 cycles of denaturing at 96°C for 30 seconds,

annealing temperature at 50°C for 15 seconds and extension at 60°C for 4 minutes. Cycle sequencing was completed with a final extension at 28°C for 1 minute and was held at 4°C in the thermo cycler.

Cycle sequencing reaction was cleaned up with the help of Just-a-Plate™ Sequencing Reaction Clean Up kit for Big Dye® Terminator clean up (AZCO Biotech, Inc.). Accordingly, the cycle sequencing product obtained was thoroughly mixed with 2µl of binding buffer supplied by the sequencing reaction clean up kit above and 25.5µl of 100% ethanol. This was subjected to centrifugation at 13000 rpm for 20 minutes at 4°C in a micro-centrifuge. Contents were pipetted out without touching the wall of the tube and kept to air dry in the dark. The dried tubes were washed with 40µl of 70% ethanol and placed on a dry block set at 65°C for 5 minutes. The purified sequencing products were eluted in 10.3µl of Hi- Di™ formamide (Applied Bio-systems Life Technologies, USA). The content was vortexed and centrifuge at 13000 rpm for 15 minutes at 4°C and denatured at 95°C for 5 min. This was immediately transferred to -20°C for about 5 minutes. All steps were carried out in the dark.

2.9.4 Analysis of electrophorogram

The sequence given in the electrophorogram was analysed with the help of Bio Edit 7.5.0.3 software and was copied into a FASTA format. The FASTA sequence obtained was BLAST against the microbial genome database available at the NCBI website (http://www.ncbi.nlm.nih.gov/) which facilitated the identification of the fungi present in the sample. However the electrophorogram which appeared distorted indicated the presence of mixed organisms. Such samples were re-PCR, cloned and sequenced.

2.9.5 Cloning experiment

The samples suspected of having mixed infections were cloned and sequenced. PCR amplified products were then gel purified using Wizard® SV Gel and PCR Clean-Up System (Promega, USA) as explained in section 2.8.6. The purified DNA segment was cloned in to pGEM®- T vector.

2.9.5.1 Cloning in to pGEM®- T vector

The purified product was ligated to the pGEM®- T vector in accordance to the user manual (Promega, 2010). pGEM®- T vector and control insert DNA tubes were briefly centrifuged to collect the contents at the bottom of the tubes. 2X ligation buffer were vortexed vigorously at about 2500 rpm before each use. The experiment was carried out in 0.5ml tubes known to have low DNA-binding capacity. Ligation reactions were set up as illustrated in Table 2.1 in section 2.8.6.2. The prepared reaction mixture was mixed by pipetting and incubated at room temperature for 1 hour followed by incubation overnight to produce maximum number of transformants (Promega, 2010).

2.9.5.3 Transforming into competent cells JM109 & BLUE/WHITE selection

The next step following ligation was the transformation of recombinant vector into the competent cells. High efficiency JM109 competent cells ($\geq 1x10^8$cfu/μg DNA) were used for the transformation reaction. These cells were provided with the pGEM®-T vector kit II (Promega, USA).

LB plates with ampicillin/IPTG/X-Gal (imMedia™ AmpBlue Invitrogen™, Life Technologies, USA) were prepared and were equilibrated to room temperature. The tubes containing the ligation reactions were centrifuged at 13000rpm for 30s and the contents collected at the bottom. 2µl of each ligation reaction was added to a sterile 1.5ml micro centrifuge tube on ice. Another tube with 0.1ng uncut plasmid were prepared and kept on ice for the determination of the transformation efficiency of the competent cells. JM109 High efficiency competent cells were placed in an ice bath until just thawed. The cells were mixed by gently flicking the tubes and excessive pipetting was avoided since the competent cells are extremely fragile.

50µl of competent cells were added in to each tube containing 2µl ligated product and 100µl of competent cells were added to the tube containing 0.1ng uncut plasmid. Gently flicked the tubes to mix and placed them on ice for 20 minutes. Cells were heat shocked for 45-50 seconds in a water bath exactly at 42°C without shaking and immediately returned the tubes to ice for 2 minutes. 950µl of S.O.C medium (Invitrogen™, Life Technologies, USA) at room temperature was added to the tubes containing cells transformed with ligation reactions and 900µl to the tube containing cells transformed with uncut plasmid. Tubes were incubated at 37°C for 1.5hours with shaking approximately at 150 rpm. 100µl of the transformed cultures were cultured on to duplicate LB/ampicillin/IPTG/X-Gal plates. Transformed control was plated with a 1:10 dilution with S.O.C. Medium (Invitrogen, USA). Plates were incubated at 37°C overnight to obtain blue and white colonies (Promega, 2010). The colonies with the insert produced white colour colonies.

2.9.5.4 Sub culturing

Approximately around 10 selected white colonies were numbered and first touched on to X-Gal/IPTG plate and then sub-cultured on to tubes containing 600µl LB broth/ampicillin. Preparation of Luria-Bertani broth (LB) with ampicillin is explained in section 2.8.6.5. The sub-cultured plate and tubes were incubated overnight at 37°C in a shaking incubator set up at 150 rpm. After overnight incubation the clear LB broth with ampicillin had a misty appearance indicating the growth of the recombined bacteria containing the target inserts. The sub-cultured plate was stored for future analysis.

2.9.5.5 Isolation of 'Plasmid DNA' from the recombined bacteria

Plasmid DNA from the sub-cultured recombined bacterial cells was isolated using Pure Yield ™ Plasmid Mini-prep System (Promega, USA). Neutralization solution (NSC) was stored at 4-8°C and the rest of the kit content was stored at room temperature. The Cell Lysis Buffer (CLC) was incubated at 35°C for 30 minutes and mixed by inverting before used. The Column Wash Solution was diluted with 95% Ethanol in accordance to user instructions in the kit manual. 100µl of the Cell Lysis Buffer (blue in colour) was added to the 600µl of sub-cultured cells and mixed by inverting the tubes 6 times. 350µl of cold Neutralizing Solution was added and was mixed thoroughly by inverting. This turned the mixture from blue to yellow colour.

The reaction mixture was centrifuged at 14000 rpm in a micro-centrifuge for 3 minutes and the supernatant transferred (~ 900µl) to a Pure Yield™ mini-column without disturbing the cell pellet. The mini-column was placed on a collection tube and centrifuged at 14000 rpm for 15 seconds. The flow-through was discarded and the mini-column was placed in the same collection tube. To this 200µl of Endo-toxin Removal Wash (ERB) was added and centrifuged at 14000rpm for 15seconds. 400µl of Column Wash solution was added and was centrifuged

at 14000rpm for 30 seconds. Finally, the mini-column was placed in a sterile 1.5ml micro-centrifuge tube and 30µl of Elution buffer added to elude the plasmid DNA out. Eluted plasmid DNA was stored in -20°C freezer until used for further analysis.

2.9.5.6 Colony PCR

Colony PCR was carried out to ensure the plasmids isolated contained the target insert of interest by amplifying a portion of the insert. Colony PCR was performed with both insert specific primers and vector specific primers to screen for the recombinant plasmids. The insert specific fungi universal primers NL1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3') and NL4 (5'- GGT CCG TGT TTC AAG ACG G-3') was used to amplify the inserted DNA fragment. This resulted in around 550 bp band when run on a 1.5% Agarose gel indicating a successful isolation of the plasmid DNA with the target DNA insert.

Similarly vector specific primers, T7 sequencing primer (5'- TAA-TAC-GAC-TCA-CTA-TAG-GG-3') and Sp6 sequencing primer (5'- ATT-TAG-GTG-ACA-CTA-TAG-3') were used to amplify a portion of the plasmid to ensure that the insert was present. Accordingly, for 5 µl of the 5x PCR reaction buffer (10mM Tris-HCl, 50mM KCl, 1.5mM $MgCl_2$, pH 8.5, 20°C) 1.5µl of 25mM $MgCl_2$ and 1.25µl 2mM dNTP' s (dATP, dGTP, dTTP and dCTP) was added. 0.6µl of each 25µM T7 forward primer and SP6 reverse primer (15pmol) and 0.5µl (1U) of Go Taq DNA polymerase (Promega, USA) was added to the above mixture along with 2µl of purified plasmid DNA. The volume of the PCR mix was adjusted to 50µl with dH_2O.

The above reaction mixtures were placed inside the ABI 2720 thermo-cycler (Applied Bio-Systems, Life Technologies USA). Initial denaturation of the genome was set at 94°C for 2 minutes, then 35 cycles of repeated heat denaturation of 30s at 94°C, annealing of the primers at 55°C for 1 minute with extension taking place at 72°C for 2.5 minutes. This reaction was concluded with a final extension at 72°C for 10 minutes and was held at 4°C. The presence of fungi was confirmed by the presence of the amplified inserted DNA fragment (which is the 28S rRNA gene) detected by running on a 1% Agarose gel which results in two bands; one band at around 550bp mark and the other band at ~ 600bp mark (primer and the insert). BstZ1 restriction enzyme digestion was also performed for the plasmids prior to sequencing.

2.9.5.7 Sequencing of plasmids for the identification of fungi

Sequencing of plasmids was carried out using automated sequencing. In summary the 10 white colonies carefully selected from the blue/white screening were sub-cultured and plasmid DNA was isolated. The plasmids DNA confirmed for the presence of the insert DNA was subjected to automated sequencing. Accordingly, 5-10 plasmid DNA was sequenced from each sample detected positive for the presence of fungus/fungi. The FASTA sequence was BLAST against the NCBI gen bank to identify the fungi present.

2.10 Detection of viruses from the placental tissue samples

2.10.1 Extraction of viral DNA

Viral DNA was extracted from the placental tissue samples using the QIAamp DNA mini kit in accordance to the user manual supplied with the kit (QIAGEN, 2010). 1g of frozen placental tissue sample excised from the original placental tissue samples were kept at room temperature for 10 minutes to thaw. Placental tissue dissolves to give placental blood when kept at room temperature for some time. 1ml of placental blood was taken into a sterile disposable syringe and filtered through sterile Millex-GP disposable 0.22 μm pore size γ-irradiated syringe filters (SIGMA, USA). To 200 μl of filtered placental blood, 20μl of proteinase K and 200μl buffer AL was added. This mixture was mixed by pulse vortexing for 15s and incubated at 56°C for 10 min. 200μl of 96-100% ethanol was added to this sample mixture which was mixed by pulse vortexing.

This mixture was carefully transferred to a QIAamp Mini spin column without wetting the rim and centrifuged at 8000rpm for 1 min. The filtrate was discarded and the mini spin column placed in a fresh collection tube. To this 500μl buffer AW1 was added and centrifuged at 8000rpm for 1 min. Similarly, 500μl of buffer AW2 was added and centrifuged at 14,000 rpm for 3 min. Finally, the spin columns were placed in a sterile 1.5 ml micro-centrifuge tube and 50μl buffer AE (10mM Tris.Cl; 0.5mM EDTA; pH 9.0) was added. After incubating for 5 minutes it was centrifuged at 8000 rpm for 1 min and the purified DNA was stored at -20°C (QIAGEN, 2010).

2.10.2 Control experiment to confirm the absence of bacterial/fungal DNA

After extracting the viral DNA it was necessary to make sure that the extracted viral DNA did not have any takeover products of bacteria or fungi. Even though the samples were subjected to filtration through 0.22μm filters restricting the passage of bacteria, fungi and human DNA it would have permitted the smaller bacterial, fungal DNA fragments to pass through. Since the next step was to carry out whole genome amplification, having carry-over products of bacteria, fungi would hinder the detection of viruses. Therefore, prior to the commencement of whole genome amplification, all viral DNA samples were PCR amplified to detect the presence of 16S rRNA and 28S rRNA genes to confirm the absence of bacteria and fungi in the DNA purified for viruses.

2.10.3 Whole genome amplification

Extracted viral DNA was then subjected to whole genome amplification using Illustra GenomiPhi^{TM} V2 DNA Amplification kit (GE Healthcare, UK). GenomiPhi^{TM} V2 DNA Amplification kit has been optimized for whole genome amplification from at least 10ng of high quality genomic DNA template. This kit contained sample buffer (green cap), reaction buffer (blue cap), enzyme mix (yellow cap) and control DNA lambda, 10ng/μl. Typical DNA yield from a GenomiPhi^{TM} V2 DNA Amplification kit was estimated to be around 4-7 μg per 20 μl reaction when started with 10ng of purified DNA. Figure 2.1 demonstrates an overview of whole genome amplification by isothermal strand displacement using the GenomiPhi^{TM} V2 DNA Amplification kit.

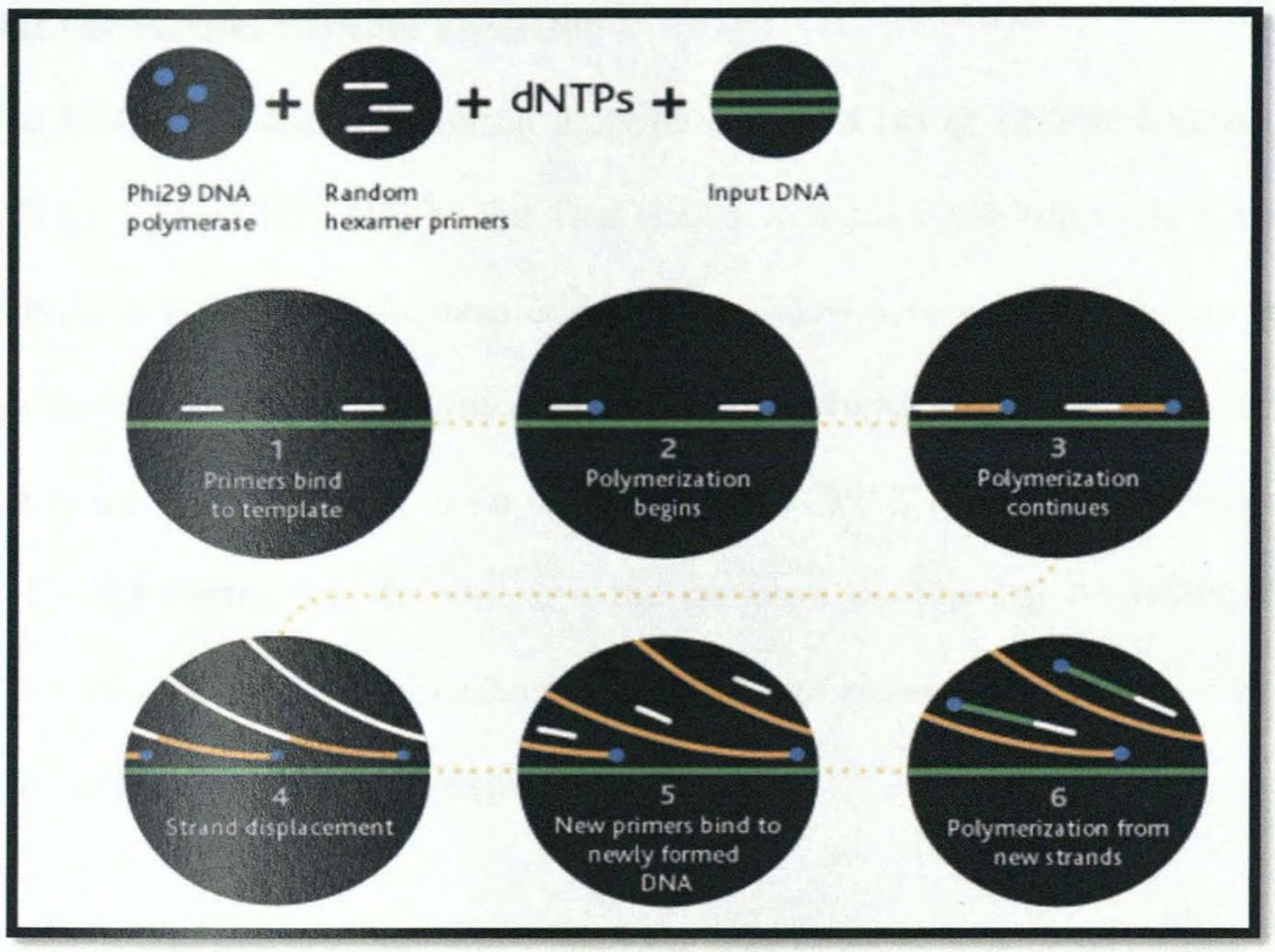


Figure 2.1 Overview of the Genomi PhiTM V2 DNA Amplification kit procedure

Phi29 DNA polymerase, random hexamer primers and dNTPs added to the input DNA and whole genome is amplified by isothermal strand displacement mechanism using the GenomiPhi^{TM} V2 DNA Amplification kit (GE Healthcare, 2006)

1µl Viral DNA was mixed with 9µl sample buffer and placed in the 2720 thermal cycler (Applied Bio-systems, USA) and heated to 95°C for 3 minutes. This was cooled to 4°C on ice. It was very important to maintain precise timings as heating the DNA for longer than 3 minutes or at higher temperatures would damage it. To the cooled mixture, 9µl of reaction buffer and 1µl of enzyme mixture was added. This mixture was maintained at < 4°C, as temperatures > 4°C would result in amplification of unwanted DNA. Finally the mixture was incubated at 30°C for 1.5 hours and was inactivated at 65°C for 10 minutes (GE Healthcare, 2006).

2.10.4 EcoRI restriction enzyme digestion

The amplified DNA was then restriction enzyme digested using 12ul/µl concentrated EcoR1 (Promega, USA). 2µl of 10X RE buffer was added to a sterile 0.2ml PCR tube and for this 0.2µl of Acetylated BSA, 10µg/µl was added. 1µl of DNA was added to this mixture along with 0.5µl of EcoR1 restriction enzymes and the final volume was made up to 20µl by adding dH_20. This mixture was subjected to 4h of incubation at 37°C and was inactivated at 65°C for 15 minutes. EcoR1 restriction enzyme cut the genome by leaving A' overhangs therefore introducing A' overhangs were not necessary. Thus the restriction enzyme digested product can be directly used for the cloning experiment.

2.10.5 Cloning experiment

2.10.5.1 Ligation into pGEM®- T vector

The above product from 2.10.4 was ligated in to pGEM®- T vector.

1.10.5.2 Transformation into competent cells JM109

The recombined vector was transformed in to JM109 competent cells and was plated in LB plates with X-gal/IPTG/amp. After overnight incubation at 37°C these plates were observed for white and blue colonies.

2.10.5.3 Blue & White selection

After overnight incubation LB plates with X-gal/IPTG/amp results in blue and white colonies. White colonies indicated the presence of viral DNA recombined in to the pGEM®- T vector.

2.10.5.4 Plasmid preparation

Ten white colonies (containing viral DNA recombined into vector) were selected and sub-cultured in LB broth with 125µl/ml ampicillin. This was incubated at 37°C overnight. Plasmids were prepared from the sub-cultured white colonies using Yield™ Plasmid Mini-prep System (Promega, USA).

2.10.5.6 Single enzyme digestion with the enzyme 'BstZ1 restriction enzyme'

Plasmids were restriction enzyme digested with the enzyme BstZ1 restriction enzyme to confirm the presence of viral genome. Refer section 2.8.6.13 for BstZ1 test procedures.

2.10.5.7 Sequencing of plasmids for the detection of viruses

Sequencing of plasmids was carried out using the same protocol followed in section 2.8.4 automated sequencing. In summary, 10 white colonies, carefully selected following blue/white screening were sub-cultured and plasmid DNA was isolated. The plasmid DNA confirmed for the presence of the insert DNA was subjected to automated sequencing. Accordingly, plasmid DNA prepared from ten white colonies was sequenced for each viral DNA sample. The FASTA sequence was BLAST against the NCBI gen bank available for all available viruses to identify the virus present in the sample.

2.11 Detection of bacteria & fungi from amniotic fluid samples

Since the molecular genetic methods for the detection of bacteria in section 2.8 concluded with positive findings for the presence of bacteria and fungi, it was decided to study further the other biological samples obtained from the same cases and control women for the presence of bacteria and fungi. For that reason DNA was extracted from the amniotic fluid samples using the QIAamp DNA mini kit (Qiagen, USA).

The 5-10ml of amniotic fluid collected at the time of delivery under aseptic conditions was centrifuged at 8000 rpm for 10 minutes. The supernatant was discarded and the pellet used for the extraction of DNA in accordance with the user manual. To 200µl of concentrated amniotic fluid, 20µl of proteinase K and 200µl buffer AL was added. This mixture was mixed by pulse vortexing for 15seconds and was incubated at 56°C for 10 min. 200µl of 96-100% ethanol was added to this sample mixture and was mixed by pulse vortexing.

This mixture was carefully transferred to a QIAamp Mini spin column without wetting the rim and was centrifuged at 8000rpm for 1 min. The filtrate was discarded and the mini spin column placed in a fresh collection tube. To this, 500µl buffer AW1 was added and centrifuged for 8000rpm for 1 min. Similarly, 500µl of buffer AW2 was added and centrifuged at 14,000 rpm for 3 min. Finally, spin columns were placed in a sterile 1.5 ml micro-centrifuge tube and 200µl buffer AE (10mM Tris.Cl; 0.5mM EDTA; pH 9.0) was added. After incubating for 5 minutes it was centrifuged at 8000 rpm for 1 min and the purified DNA stored at -20°C (QIAGEN, 2010).

16S rRNA gene was amplified using polymerase chain reaction as described in section 2.8.2 to detect the presence of bacteria. After running on a gel the samples which resulted in a 500bp band indicating the presence of bacterial 16S rRNA gene unique to all species were then subjected to automated sequencing and were BLAST against the NCBI GenBank database to identify the bacteria present in the sample. As described in section 2.8.5, after analysing the electrophorogram the samples indicating mixed infection was cloned and further studied.

Likewise the extracted DNA from amniotic fluid samples was further amplified to detect the presence of 28S rRNA gene common to all fungi as described in section 2.9. The samples resulting in a 550bp band indicating the presence of fungi was subjected to automated sequencing.

2.12 Detection of bacteria & fungi from blood samples

DNA was extracted from the blood samples using the QIAamp DNA mini kit according to the blood and body fluid protocol (Qiagen, USA). 20µl of proteinase K was added to 200µl of blood and to this 200µl buffer AL was added. DNA was extracted from blood samples as described in section 2.11.

The 16S rRNA gene was amplified using PCR as described in section 2.8.2 to detect bacteria. After running on a gel the samples which resulted in a 500bp band indicating the presence of bacterial 16S rRNA gene unique to all species were subjected to automated sequencing and were BLAST against the NCBI GenBank database to identify the bacteria present in the sample. As described in 2.8.5, after analysing the electrophorogram, the samples indicating mixed infection were cloned and individual colonies studied further.

Similarly the extracted DNA from blood samples was further amplified to detect the presence of 28S rRNA gene common to all fungi as described in section 2.9. The samples resulting in a 550bp band indicating the presence of fungi was subjected to automated sequencing.

2.13 Detection of bacteria & fungi from urine samples

The 5-10ml of urine collected under aseptic conditions at the time of delivery was centrifuged at 8000 rpm for 10 minutes. The supernatant was discarded and the pellet used for the extraction of DNA in accordance with the QIAamp DNA mini kit (Qiagen, USA) user manual. To 200µl of concentrated urine 20µl of proteinase K and 200µl buffer AL was added. The DNA extracted from urine samples were also tested for the presence of bacteria and fungi by the amplification of 16S and 28S rRNA gene. The positive samples were cloned, sequenced and identified by blasting to the GenBank database available at the NCBI website.

PHASE 2

2.14 16S Metagenomics Next Generation Sequencing

In phase 2, placental tissue samples obtained from 55 women with pre-eclampsia and 55 normotensive women were further analysed on an Illumina MiSeq next generation sequencing platform for the detection and identification of bacteria. The first step in 16S metagenomics was to amplify the hyper variable regions of the 16S ribosomal RNA gene of bacteria by PCR. The whole 16S rRNA gene which is 1542bp in length is amplified by PCR using universal primers; BSF8/20 as the forward primer and BSR1514/20 as the reverse primer (Cai et al., 2003). The 16S metagenomics is known as bacterial metagenomics due to its great ability to detect and identify bacteria with the highest sensitivity and specificity.

2.14.1 DNA extraction

DNA was extracted from the placental tissue samples of the 55 women with pre-eclampsia and 55 normotensive women using QIAamp DNA mini kit (Qiagen, USA) in accordance to the 'Tissue protocol' of the user manual supplied with the kit. All extracted DNA was stored at -20°C. The detailed DNA extraction procedures by means of Qiagen QIAamp DNA mini kit is described in section 2.8.1.

2.14.2 16S rRNA whole gene amplification using PCR

A set of universal primers 16S rRNA BSF8/20 (5'- AGA GTT TGA TCC TGG CTC AG -3') and 16S rRNA BSF1541/20 (5'- AAG GAG GTG ATC CAG CCG CA-3') was used to amplify the complete 16S ribosomal RNA gene unique to all bacteria.

To 5 µl of the 5x PCR reaction buffer (10mM Tris-HCl, 50mM KCl, 1.5mM $MgCl_2$, pH 8.5, 20°C) 1.5µl of 25mM $MgCl_2$ and 1.25µl 2mM dNTP' s (dATP, dGTP, dTTP and dCTP) was added. 0.3µl of each 50µM forward and reverse primers (15pmol) and 0.2µl (1U) of Go Taq DNA polymerase (Promega, USA) was added to prepare the PCR master mix. 3µl of purified DNA were finally added to the master mix and the final volume was made to 25µl using dH_2O. The 16S rRNA gene was amplified by PCR in an ABI 2720 thermo-cycler (Applied Bio-Systems, Life Technologies USA). Initial denaturation of the genome was set at 95°C for 5 minutes, then 35 cycles of repeated heat denaturation at 94°C for 30s, annealing of the primers were set at 61.5°C for 30s and the extension taking place for 60s at 72°C. The reaction was completed with the final extension at 72°C for 12 minutes and was held at 4°C. The presence of bacteria was confirmed by the presence of the 1500bp band detected by gel electrophoresis.

2.14.3 Purification of amplified DNA template

Equal volume of membrane binding solution supplied with the Wizard® SV gel and PCR clean-up system (Promega, USA) and the PCR product was added to a sterile 1.5ml tube. This mixture was transferred on to SV mini column supplied with the kit and incubated at room temperature for 1 minute. It was centrifuged at 14,000 rpm for 1 minute and the resulting flow through was discarded. 700µl of membrane wash solution was then added and centrifuged for 14000 rpm for 1 minute. The column was repeatedly washed with 500µl of membrane wash

solution which has pre added 95% ethanol. After centrifuging for 5 minutes at 14000 rpm, the mini column was placed at room temperature for 5 minutes to evaporate any remaining ethanol. 50µl of nuclease free water was added and left at room temperature for 1 minute. The purified DNA was then eluted by centrifuging at 14000 rpm for 1 minute. The purified DNA eluted was stored in a -20°C freezer until sequenced by next generation sequencing technology.

2.14.4 Metagenomics NGS using Nextera XT DNA sample preparation kit

2.14.4.1 Quantification of the DNA library

DNA is fragmented using enzymes in the Nextera XT DNA sample preparation kit. The ultimate success of the assay strongly depends on the quality and quantity of the input DNA. Accordingly prior to using the Nextera XT DNA sample preparation kit, the amount of DNA in the samples were quantified using the Qubit® dsDNA BR assay system (Invitrogen, 2011).

The Qubit® dsDNA BR assay kit was used with the Qubit® 2.0 Fluorometer for the quantification of DNA. Qubit® working solution was made by diluting the Qubit® dsDNA BR reagent 1:200 in Qubit® dsDNA BR buffer. 10µl of each Qubit® standard was added to 190µl of Qubit® working solution to make up the final volume to 200µl, and was mixed by vortexing for 2-3 seconds. 5µl of sample was added to 195µl of Qubit® working solution and was mixed by vortexing for 2-3 seconds. All tubes were incubated at room temperature for 2 minutes. 'DNA' was selected on the home screen of the Qubit® 2.0 Fluorometer and the 'dsDNA Broad range' was selected as the assay type. The 'new calibration' option was selected on the standard screen. The tube containing Standard #1 was inserted into the Qubit® 2.0 Fluorometer and was 'read'. Next, the tube containing Standard #2 was inserted into the Qubit® 2.0 Fluorometer

and was read. Similarly, the concentration of the rest of the samples was measured in µg/ml. This value corresponds to the concentration after the samples were diluted in the assay tube. The Qubit® 2.0 Fluorometer calculates the original sample concentration based on the measured assay concentration (Invitrogen, 2011).

2.14.4.2 Tagmentation of genomic DNA

The input DNA was 'tagmented' (tagged and fragmented) by the Nextera XT 'transposomes'. The Nextera XT transposome simultaneously fragmented the input DNA and added adapter sequences to the ends. Amplicon Tagment Mix (ATM), Tagment DNA buffer (TD) supplied with the Nextera XT kit and input DNA was removed from -20°C storage and allowed to thaw on ice while the Neutralize Tagment Buffer (NT) was kept at room temperature. When there were signs of precipitation in the NT buffer, it was necessary to vortex at about 2500 rpm until all particles were re-suspended.

All reagents were adequately mixed by gently inverting the tubes 3-5 times followed by a brief spin in a micro-centrifuge. A new 96 TCY plate was labelled as NTA (Nextera XT Tagment Amplicon Plate). 10µl of TD buffer was added to each well to be used in this assay by changing tips between samples. 5µl of input DNA at 0.2ng/µl was added to each sample well of the NTA plate. 5µl of ATM was added to the wells containing input DNA and TD buffer and using a multi-channel pipette mixed by gentle pipetting 5 times up and down. The NTA plate was covered with a micro seal 'B' and centrifuged at 280xg at 20°C for 1 minute. The NTA plate was placed in a thermo cycler and the following programme was run; 55°C for 5 minutes and holding at 10°C. Once the samples reached 10°C, contents in the NTA plates were immediately neutralized (Illumina, 2012a).

2.14.4.3 Neutralize NTA

The micro seal 'B' was carefully removed and 5µl of NT buffer was added to each well of the NTA plate by changing tips in between samples, and gently mixed using a multi-channel pipette. The NTA plate was again covered by the micro seal 'B' and centrifuged at 280xg at 20°C for 1 minute. The NTA plate was placed at room temperature for 5 minutes and proceeded to the PCR amplification step (Illumina, 2012a).

2.14.4.4. PCR Amplification

Nextera PCR Master Mix (NPM) and the index primers were removed from the freezer and thawed at room temperature. After all reagents were completely thawed, the tubes were mixed gently and the tubes briefly centrifuged 14000 rpm for 30s in a micro centrifuge. Index 1 (i7) primers with the orange cap were arranged horizontally according to the order so that N701 was in column 1 and N706 was in column 6. Index 2 (i5) primers with the white cap were arranged vertically according to the order, in such a way that S501 was in row A and S504 was in row B (figure 2.2).

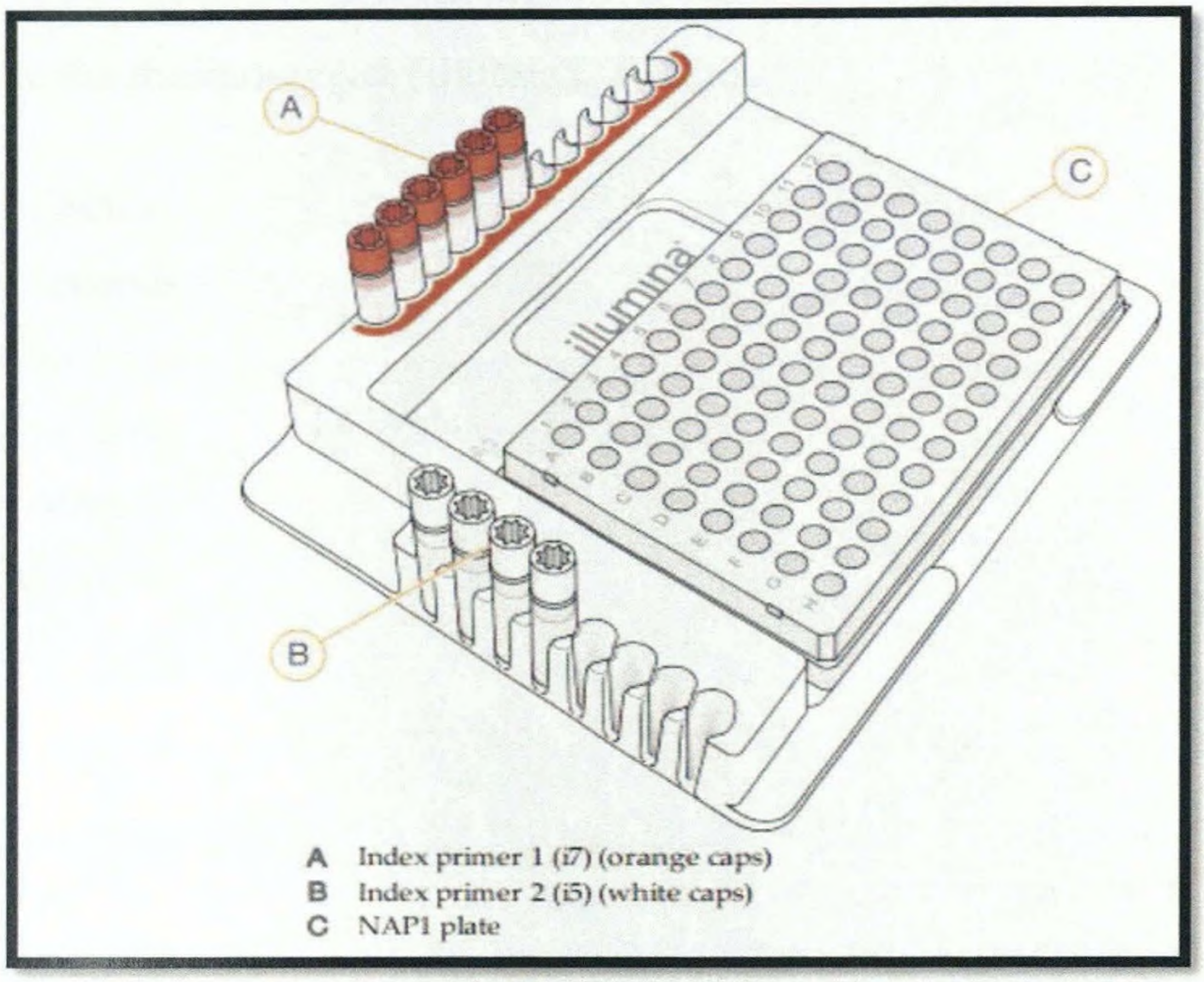


Figure 2.2 TruSeq Index Plate Arrangements

TrueSeq Index Plate fixture is a platform available to place the NTA plate along with the two index primers, for easy handling and processing of samples (Illumina, 2012a)

The 'NTA plate' was placed in the 'True Seq Index Plate Fixture' platform as illustrated in figure 2.2. 15µl NPM was added to each well of the NTA plate. Using a multichannel pipette, 5µl of the index 2 (i5) primers with the white caps were added to each column of the NTA plate. 5µl of index 1(i7) primers with the orange caps were also added to each row of the NTA plate. To avoid index cross contamination, the original white and orange caps were discarded and were replaced with a set of new white and orange caps provided with the kit. The plate was sealed using the micro seal 'A' using a rubber roller and was centrifuged at 280 x g at 20°C for 1 minute.

After the centrifugation step, DNA was amplified using the following programme by placing the NTA plate inside the thermo-cycler (Illumina, 2012a).

- 72°C for 3 minutes
- 95°C for 30 seconds
- 95°C for 10 seconds
- 55°C for 30 seconds
- 72°C for 30 seconds
- 72°C for 5 minutes
- Hold at 10°C

12 cycles

2.14.4.5 PCR clean up

PCR clean-up and the purification of the DNA library was carried out using the AMPure XP beads kit. This step removes very short library fragments from the population. A new MIDI plate was labelled as 'Clean Amplified Plate' (CAA). Consequently the NTA plate was centrifuged at 280 x g for 1 minute at 20°C and 50µl of the PCR product from the NTA plate was transferred to the CAA plate. Next the AMPure XP beads were vortexed at 2500 rpm for 30 seconds to ensure that the beads were evenly dispersed. Appropriate volume of beads was then added to a trough and 30µl transferred to each well of the CAA plate. 60µl of 1.8x AMPure XP was used in order to obtain a 300-500bp amplicon size.

The above reaction was gently mixed by pipetting up and down 10 times and incubated for 5 minutes at room temperature without shaking. The plate was placed on a magnetic stand for 2 minutes. The supernatant was removed and discarded while leaving the CAA plate on the magnetic stand. While pipetting if any beads accidentally entered the tip, it was dispensed back to the plate which was left on the magnetic stand for 2 minutes until the supernatant cleared.

The beads were washed by adding 200µl of freshly prepared 80% ethanol to each sample on the plate and were incubated for 30 seconds on the magnetic stand. The supernatant was carefully removed and discarded. The washing step was repeated twice again by adding 200µl of freshly prepared 80% ethanol to each sample well and the plate incubated for 30 seconds on the magnetic stand as before. The supernatant and excess ethanol was removed using a P20 multichannel pipette.

The beads were air dried for 15 minutes while leaving the CAA plate on the magnetic stand. The CAA plate was then removed from the magnetic stand and 52.5µl of re-suspension buffer (RSB) added to each well. This was gently mixed by pipetting up and down 10 times and incubated for 2 minutes at room temperature. Again the plate was placed on the magnetic stand for 2 minutes till the supernatant was cleared. Another new TCY plate was named as CAN (Clean Amplified NTA plate) and 50µl of the supernatant from the CAA plate was carefully transferred on to the CAN plate (Illumina, 2012a).

2.14.4.6 Library normalization

This process normalizes the library to ensure more identical library representation in the pooled samples. Accordingly, LNB1 (Library Normalization Beads 1) were vigorously vortexed at 2500 rpm for at least 1 minute to ensure the beads were well re-suspended and no pellet was found at the bottom of the tube. A new MIDI plate was labelled as LNP (Library Normalization Plate). Using a P20 multichannel pipette and fine tips, 20 µl of the supernatant from the CAN plate was transferred on to the LNP plate. For the 7 samples, 360µl of LNA1 (Library Normalization Additives 1) was added and mixed by pipetting up and down 15-20 times until properly suspended.

66.6µl of LNB1 was transferred to the tubes containing LNA1 and mixed by inverting for 15-20 minutes. 45µl of the resulting LNA1/LNB1 bead mix was added to the LNP plate containing the libraries. Changing tips in between samples was not considered necessary at this stage. The LNP plate was sealed by a micro seal 'B' and placed on a micro plate shaker at 1800 rpm for 30 minutes. Afterwards this it was placed on a magnetic stand for 2 minutes to clear the supernatant. With the help of a multichannel pipette 80µl of the supernatant was carefully pipetted out and discarded from the LNP plate whilst the plate remained on the magnetic stand.

The LNP plate was then removed from the magnetic stand and the beads were washed with LNW1 (Library Normalization Wash 1) by adding 45µl of LNW1 to each sample well and were sealed with a micro seal 'B'. This LNP plate was further placed on a micro plate shaker at 1800 rpm for 5 minutes and placed back on a magnetic stand for 2 minutes. Once the supernatant that appeared cleared it was pipetted out and discarded. The washing step with LNW1 was repeated.

The LNP plate was then removed from the magnetic stand and 30µl of 0.1N NaOH added to each well to elute the samples. The plate was sealed using the micro seal 'B'. The LNP plate was then placed on a micro plate shaker at 1800 rpm for 5 minutes until completely re-suspended. Meanwhile, SGP (StoraGe Plate) barcode sticker was pasted on a new 96 well PCR plate. 30µl of LNS1 (Library Normalization Storage Buffer 1) was added to each well to be used in the GSP plate. The LNP plate was placed on a magnetic stand for 2 minutes until the supernatant cleared. 30µl of the supernatant was transferred from the LNP plate to the GSP plate which was sealed with micro seal 'B'. This plate was centrifuged at 1000 x g for 1 minute (Illumina, 2012a).

2.14.4.7 Library pooling for MiSeq sequencing

For cluster generation and sequencing, equal volumes of normalized libraries were combined, diluted in hybridization buffer and heat denatured prior to MiSeq sequencing. To do so, a heat block suitable for 1.5ml tubes was adjusted to 96°C. MiSeq reagent cartridge was removed from the freezer and allowed to thaw at room temperature. An ice bucket was prepared in the proportion of 3:1 ice to water.

The GSP plate was centrifuged at 1000xg for 1 minute at 20°C for condensation. Using a P200 multichannel pipette, 5µl of each library to be sequenced was transferred from the plate, column by column, to a PCR eight tube strip. A fresh 1.5ml tube was labelled as PAL (Pooled Amplicon Library). The contents of the PCR eight tube strips was combined and transferred to the PAL tube and mixed well. Another fresh 1.5ml tube was labelled as DAL (Diluted Amplicon Library). 588µl of Hybridization buffer (HT1) was added to DAL tube. 13µl of PAL was transferred from PAL to the DAL tube containing HT1. The DAL tube was vortexed at high speed at 2500 rpm and incubated at 96°C for 2 minutes. After incubation, the DAL tube was inverted 1-2 times and immediately placed on ice for 5 minutes. The DAL was then loaded onto the 'Load Samples' reservoir in the 'MiSeq reagent cartridge' and sequenced in the Illumina MiSeq Bench-top Genetic Analyser (Illumina, 2012a). The quality of the library pool was further confirmed with the help of 'Agilent High Sensitivity DNA kit'.

2.14.4.8 Quality check of library pool using Agilent High Sensitivity DNA kit

Quality of the library pool was checked using Agilent High Sensitivity DNA kit (Agilent Technologies, Germany). The high sensitivity DNA dye concentrate and high sensitivity DNA gel matrix were allowed to equilibrate to room temperature for 30 minutes. The gel dye mix was prepared by adding 15µl of high sensitivity DNA dye concentrate to the high sensitivity DNA gel matrix vial. The solution was transferred to the spin filter and was centrifuged at 2240 x g for 10 minutes, stored at 4°C.

Next step was to load the gel dye mix onto the chip. Accordingly, the gel dye mix was equilibrated to room temperature for 30 minutes and a new high sensitivity DNA chip was placed on the priming station. 9µl of gel dye mix added onto the well-marked as '' on the chip. The plunger was positioned at 1ml and the priming chip station was closed. Then the plunger was pressed until it was held by the chip. After 60s the chip was released and after 5s the plunger was slowly pulled back to the 1ml position. The chip priming station was opened and 9µl of gel dye mix was pipetted into the wells marked as ''.

5 µl marker green was pipetted into all samples and ladder wells. Then 1 µl of DNA ladder in yellow was added to the well-marked as ''. 1 µl of the sample was added to the wells and 1 µl of marker was added to the unused wells. The chip was placed horizontally in the adapter and vortexed at 2500 rpm for 1 min. This chip was run on the Agilent 2100 Bio-analyzer (Agilent Technologies, Germany) to measure the quanlity of DNA library.

2.14.4.9 Sequence analysis using 'MiSeq Reporter' software

Sequences were analysed using the 'MiSeq Reporter' analysis software which is available in the Illumina MiSeq Genetic Analyser. The classification results up to the 'Genus' level and will provide the numbers and percentages of the total microbiome of all samples pooled. The results were viewed over the online tool option called 'BASE SPACE'.

2.14.4.9 Species level analysis using other metagenomics analysis software's

The MiSeq reporter software available within the MiSeq Genetic Sequencer is designed to classify only up to genus level. Therefore, other metagenomics analysis software's such as 'GALAXY PROJECT' and 'MOTHUR' were used to classify the samples up to species level. GALAXY PROJECT (http://galaxyproject.org/) and MOTHUR (http://www.mothur.org/) used in this research project for the species level classification is an open, web based platform (Jones et al., 2009, Preeclampsia_Foundation, 2013). Species level classification was carried out using the Mothur software (open source from the University of Michigan) which allowed the identification at species level. This software seeks the closest template for each candidate using kmer searching and k-nearest neighbor consensus approach as search method. Needleman-Wunsch alignment method is used for aligning the templates to the GreenGenes, NCBI Taxonomy (Bacteria) and SILVA (Bacteria) databases to identify the bacteria up to the species level.

2.15 16S Independent Metagenomics Next Generation Sequencing

2.15.1 DNA extraction

The aim of the phase 2 study was to detect and identify bacteria from placental tissues utilising both 16S metagenomics and 16S independent metagenomics. What is characterised by 16S independent metagenomics is the whole genome metagenomics without the use of 16S universal primers to amplify the bacteria. The only way to perform 16S independent metagenomics is to amplify the whole genome of bacteria. Therefore it is necessary to make sure that only bacterial DNA is extracted from the sample.

Accordingly, the DNA was extracted from the placental tissue samples using 'MolYsis Basic' kit from the Molzym GmbH & Co.KG, Germany. As mentioned above, molecular analysis of infectious bacteria in clinical samples could be severely disturbed by high background of host DNA. MolYsis Basic removes the background of host DNA and thereby increases the efficiency of the molecular analysis of bacteria from the human placental tissue sample.

MolYsis Basic enables the preparation of bacterial DNA from the human samples in two steps. i) By the addition of chaotropic buffer which lyses the host cells and leaves the bacterial cells unaffected. Buffer CM supplied with the kit is a chaotropic buffer and would lyse the human cells. ii) The DNA released from host cells will be degraded by Molzyme's chaotrope-resistant MolDNase A. Thereafter, bacterial cells will be treated with BugLysis reagent to degrade the cell walls of gram negative and gram positive bacteria. The DNA obtained will be further purified using the QIAamp DNA mini kit of Qiagen (MOLZYM, 2011).

The isolation of bacterial DNA from the placental tissues was performed according to the user manual supplied with the MolYsis Basic kit of Molzyme, Germany. Based on such guide lines, 200µl of placental blood was pipetted into a sterile 2ml tube and 50µl of buffer CM (supplied with the kit) was added to the tube. Mixed by vortexing at full speed at 2500 rpm for 10seconds and was allowed to stand on the bench at room temperature for 5 minutes.

50µl buffer DB1 and 10µl MolDNaseA was added to the lysate which was vortexed at 2500 rpm immediately for 10seconds. This was allowed to stand for 15 minutes at room temperature. The step ensures the degradation of human DNA. The bacterial cells were then harvested by centrifuging in a bench top micro centrifuge at ≥13,000 rpm for 5 minutes. The supernatant was carefully removed by pipetting.

To this 80µl of buffer RL was added and the pellet re-suspended by pipetting in and out. This was further vortexed at 2500 rpm for 10s to homogenize. Finally 20µl of Bug-Lysis solution was added and vortexed at 2500 rpm for 10s and further incubated at 37°C for 30 minutes at 100 rpm to degrade the bacterial cell wall. 100 µl of buffer RL was added to re-suspend the bacterial DNA. Then the Bacterial DNA was isolated and purified using the QIAmp DNA mini kit (Qiagen, USA) as described previously in section 2.8.1.

2.15.2 Quantification of DNA

Isolated bacterial DNA was quantified using Qubit® dsDNA BR assay system as explained in section 2.14.4.1. The results were further confirmed by performing 16S rRNA gene amplification PCR assay to amplify the 16S ribosomal RNA gene of bacteria present in the sample.

2.15.3 Next Generation Sequencing

The confirmed bacterial DNA was to be processed by Next Generation Sequencing using Illumina MiSeq Genetic Analyser. However, the bacterial DNA isolated from the above method using MolYsis kit was not adequate to be sequenced on a NGS platform. Accordingly, the best possible method to carry out metagenomics next generation sequencing was to follow the 16S Metagenomics approach in which the whole gene of 16S ribosomal RNA gene was amplified and sequenced using next generation sequencing.

2.16 Quality control considerations

From the very first day the research project commenced, priority was given to the quality and validity of the experiment. As a quality control precaution, sterility was maintained right throughout the research project and aseptic techniques were followed at all times. All samples were collected at the time of caesarean section under stringent aseptic measures with the extensive support of experienced doctors and nurses. Samples were stored in sterile containers and were immediately transported to the laboratory and were stored in -80°C.

All pre-polymerase chain reaction (pre-PCR) experiments were carried out inside a class II bio-safety cabinet using barrier tips which were UV sterilized prior to use. All tips and glassware taken for the experiment were sterilized using an autoclave. To ensure the accuracy of the results, the PCR experiments were carried out in batches of 5-10, along with a positive control and a negative control. The positive control was pre-tested and proven to be positive. The negative control was ddH_2O. In case of detecting an unwanted band in the negative control it was considered a contamination and the whole experiment batch was repeated. Positive samples were repeated twice for confirmation prior to reporting. Colony PCR was performed to ensure the gene inserted in to the vector was the target DNA of interest which was further confirmed through restriction enzyme digestion. This would confirm that the colony sequenced contained the target 16S/28S rRNA insert of interest. After the viral DNA purification 16S/28S rRNA genes were amplified by PCR as an internal experiment to ensure there was no carry-over of bacterial or fungal gene fragments. All contaminated gloves, masks, tissue papers, tips, tubes, plastic ware were disposed per laboratory safety guidelines.

3 RESULTS

3.1 Study population

3.1.1 Recruitment of cases and controls

Out of the 228 women referred to the study 55 women meeting the inclusion criteria were recruited as cases. Even if one or more exclusion criteria were present they were excluded/withdrawn from the study. Table 3.1 provides a summary of the patients who were referred but not recruited to the study. The main reasons for women with pre-eclampsia being excluded or withdrawn from the study was that they were multiparous and not meeting the proteinuria criteria. All women who volunteered for the study were recruited prior to the delivery and samples were collected at the time of delivery via caesarean section aseptically.

Table 3.1 Summary of the women who were referred but not recruited to the study

	No (%)
Total referred	**228**
Reasons for exclusion	
Gestation diabetes	1 (0.4)
Chronic hypertension	1 (0.4)
Twin delivery	1 (0.4)
Fetal abnormalities	2 (0.8)
Both gestation diabetes & failed proteinuria	2 (0.8)
Normal delivery	2 (0.8)
Un matched as case/control	6 (2.6)
Unable to obtain samples	9 (3.9)
Failed proteinuria criteria	20 (8.7)
Multiparous	74 (32.4)
Total recruited	**55 (Cases)** **55 (Controls)**

Indications for the 55 normotensive pregnant women to undergo caesarean section are indicated in the table below. Accordingly, all normotensive pregnant women underwent caesarean section due to medically uncomplicated reasons such as primi-breech, maternal request, subfertility, large baby, head not engaged, unfavourable cervix, fetal distress and presence of fibroids.

Table 3.2 Indication for caesarean section for the 55 normotensive pregnant women

Indication for caesarean section	No (%)
Breech	21 (38.1)
Maternal Request	14 (25.4)
Subfertility	6 (10.9)
Large baby	5 (9.0)
Unfavourable cervix	4 (7.2)
Fetal distress	2 (3.6)
Head not engaged	2 (3.6)
Fibroids	1 (1.8)
Total	**55**

3.1.2 Racial distribution of both cases and controls

As indicated in figure 3.1 both women with pre-eclampsia and normotensive pregnant women share a similar racial distribution. Demographic distribution of women with pre-eclampsia and the normotensive pregnant women recruited for the study is detailed below.

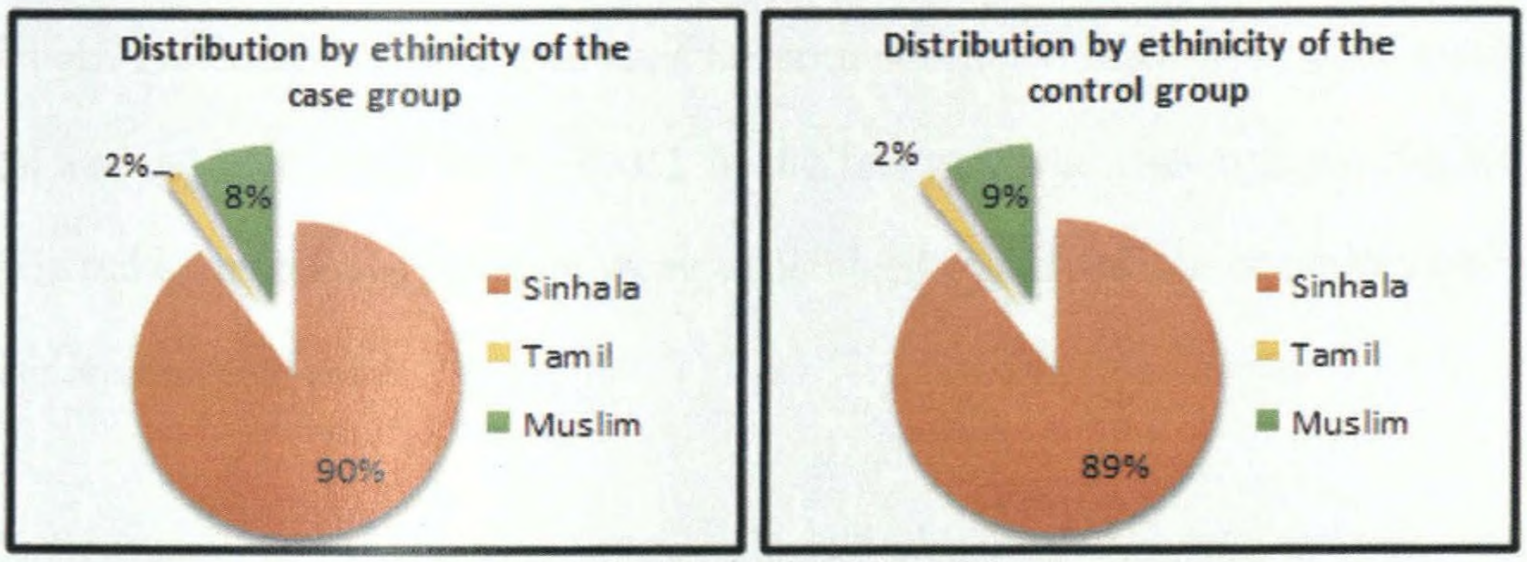


Figure 3.1 Racial distribution of controls recruited to the study

Distribution of ethnicity of women recruited to the study in both case and control groups were very much similar. Where 90% of women with pre-eclamsia recruited to the study was Sinhala, 2% Tamil and 8% Muslim. In the control group, 89% of women recruited were Sinhala, 2% Tamil and 9% were Muslim.

3.1.3 Phenotypic features of cases and controls on their first prenatal visit

Both women with pre-eclampsia and normotensive pregnant women were primiparous. None of them had ever smoked. As shown in table 3.3, there was no significant difference between the two groups of women recruited for the study at the time of antenatal booking. The mean age for the cases was 27.5 and controls were 28.0. The mean body mass index for the case group was 22.2 and the matched control group had a mean body mass index of 22.8. Accordingly, Pre-eclampsia case group and the normotensive control group were matched for maternal age and body mass index (BMI). At the first antenatal visit both women with pre-eclampsia and normotensive pregnant women had blood pressure in the normal range and had no evidence of proteinuria.

Table 3.3 Comparison of the phenotypic features of both cases and controls

		Women with Pre-eclampsia (n=55)	Normotensive women (n=55)	p- value
Mean Age	(SD)	27.5 (5.4)	28.0 (5.3)	1.000
Mean body mass index‡ (kg/m2)	(SD)	22.2 (3.1)	22.8 (3.0)	0.607
Gestation (weeks)†	M(iqr)	9.4 (8.0-13.6)	8.4 (7.4-13.0)	0.512
Blood pressure				
Mean Systolic (mmHg)	(SD)	110.2 (10.4)	111.8 (8.1)	0.521
Mean Diastolic (mmHg)	(SD)	73.1 (7.8)	72.7 (5.6)	0.188

M (iqr), median (interquartile range); SD, standard deviation, †7 pregnancies were booked after 20 weeks of gestation. ‡ The weight and height were measured after 20 weeks in un-booked women.

3.1.4 Comparison of the demographic characters of cases and controls

The disease aetiology may play a significant role in the disease diagnosis and management. Characteristics of women with pre-eclampsia (case group) at delivery is summarised in table 3.4. The mean gestational age at delivery was 36.0, where 36 (65.4%) women delivered pre-term (before 37 weeks of gestation) and 18 (32.7%) of women delivered before 34 weeks.

Table 3.4 Comparison of the gestational ages at the time of delivery

		Women with pre-eclampsia	**Normotensive pregnant women**
Gestation at delivery (weeks)	**M(iqr)**	**36.0 (33.0-37.2)**	**38.2 (38.0-38.5)**
Before 34 weeks	n (%)	18 (32.7%)	0
34 to 36.6 weeks	n (%)	18 (32.7%)	0
Before 37 weeks (Pre-term delivery)	n (%)	36 (65.4%)	0
After 37 weeks (Delivery at Term)	**n (%)**	**19 (34.5%)**	**55 (100%)**

M (iqr), median (interquartile range); n (%), number (percentage)

As discussed in chapter 1, pre-eclampsia is liable for severe complications and is a life threatening condition. Figure 3.2 indicates that even though all normotensive pregnant women delivered at term (after the 37 weeks of gestation), more than 65.4% of women with pre-eclampsia delivered pre-term before 37 weeks of gestation. The median gestation age at delivery for the normotensive pregnant women remained at 38.0 (38.0-38.5). All deliveries related to normotensive women were uncomplicated compared to the women with pre-eclampsia.

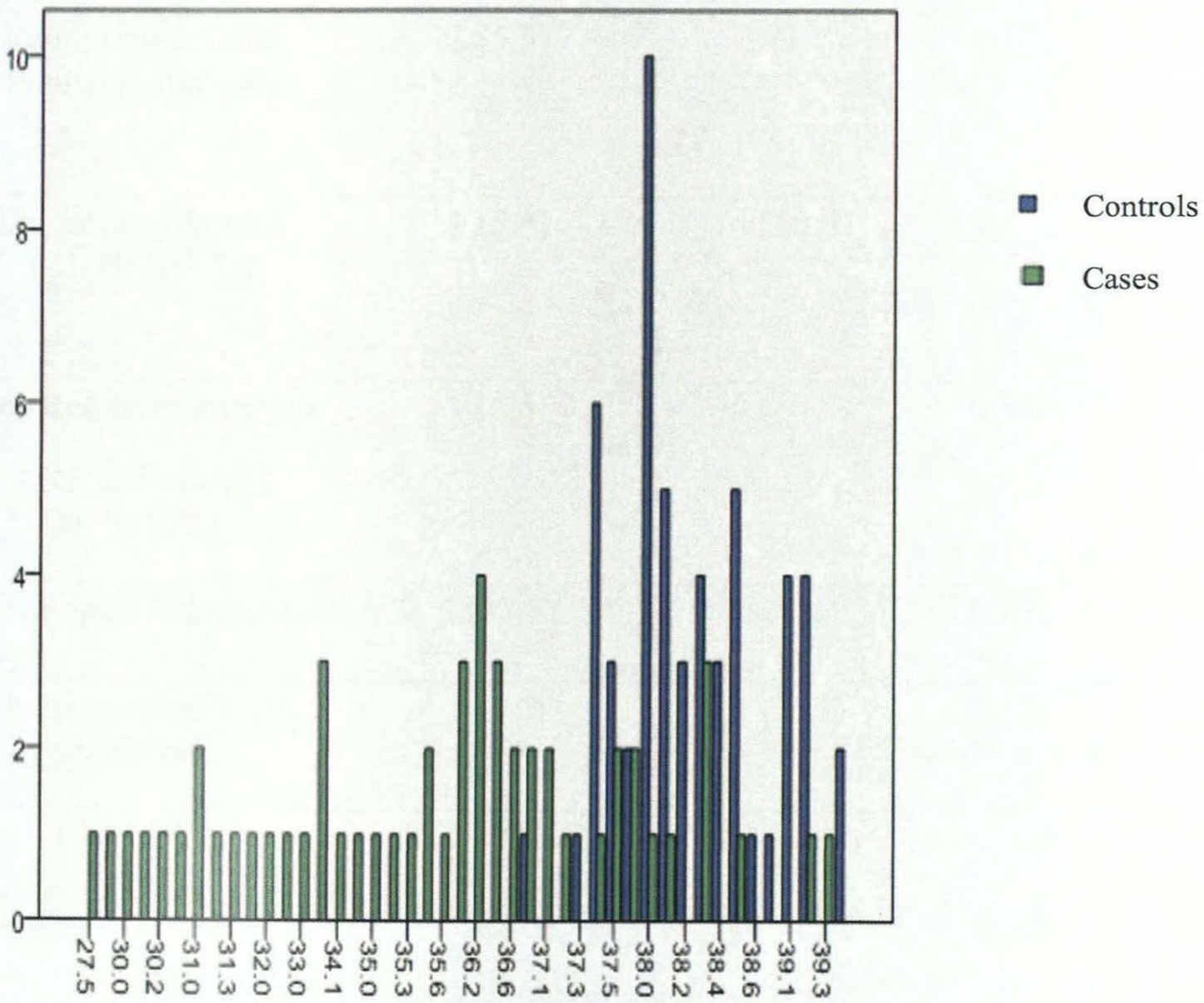


Figure 3.2 Gestation at delivery for the cases and controls

More than half of women with preeclampsia (cases) had preterm deliveries, where some of them even delivered as early as 27.5 weeks. All normotensive pregnant women (controls) delivered at term after 37 weeks of gestation.

Table 3.5 Complications observed in women with pre-eclampsia

Complications	Early on set (<34 weeks) (n = 18) No (%)	Late onset (≥ 35 weeks) (n = 37) No (%)	p-value	PE n = 55 No (%)
SBP ≥ 160mmHg	13 (72.2)	17 (45.9)	0.068	30 (54.5)
DBP ≥ 110mmHg	7 (38.8)	10 (27.0)	0.381	17 (30.9)
Proteinuria (≥3+HCT)	12 (66.6)	16 (43.2)	0.107	28 (50.9)
Renal impairment requiring dialysis	1 (5.5)	1 (2.7)	0.572	2 (3.6)
Thrombocytopenia (<100x10^9 /L)	1 (5.5)	4 (10.8)	0.534	5 (9.0)
Elevated liver enzyme (AST and/or ALT ≥ 70 U/L)	1 (5.5)	4 (10.8)	0.534	5 (9.0)
Developed Eclampsia	5 (27.7)	3 (8.1)	0.054	8 (14.5)
Developed HELLP syndrome	1 (5.5)	1 (2.7)	0.194	2 (3.6)
EM/LSCS	15 (83.3)	17 (45.9)	0.008	32 (58.1)
Admitted to Intensive care unit	8 (44.4)	5 (13.5)	0.011	13 (23.6)
Maternal death	0	0	-	0

SBP, systolic blood pressure; DBP, diastolic blood pressure; AST, aspartate aminotransaminase; ALT, alanine aminotransaminase;HCT, the urine protein heat coagulation test.

Table 3.5 compares the complications observed in women with early onset and late onset of pre-eclampsia. It is evident that women with early onset pre-eclampsia have a higher risk of developing pregnancy complications compared to the women with late onset pre-eclampsia. 18 of the 55 (32.7%) women with preeclampsia in our study population developed early onset pre-eclampsia. Under these circumstances it is apparent that, early onset pre-eclampsia needs to be carefully observed as it may lead to severe maternal and perinatal morbidity and mortality.

As shown in table 3.5, 13 of 18 (72.2%) women with early onset pre-eclampsia developed severe systolic blood pressure of ≥ 160mmHg and 12 (66.6%) developed proteinuria of ≥ 3+ HCT. In this group, women with early onset pre-eclampsia were 70% vulnerable to the risk of developing a severe form of the disease. Furthermore women with early onset pre-eclampsia were three times more likely to develop eclampsia compared to the women with late onset pre-eclampsia (P= 0.05). 15 out of the 18 (83.3%) women with early onset pre-eclampsia underwent emergency caesarean sections (P= 0.008).

In summary, 30 (54.5%) of 55 women with pre-eclampsia had ≥ 160mmHg, 17 (30.9%) had ≥ 110mmHg and 28 (50.9%) proteinuria. 32 (58.1%) of 55 women with pre-eclampsia underwent EM/LSCS. However, all normotensive pregnant women underwent EL/LSCS and had no pregnancy complications.

Table 3.6 illustrates the rising blood pressure of women with pre-eclampsia. When considering the highest recorded blood pressure of women with pre-eclampsia for the study, 52 (94.5%; n=55) had a ≥ 30mmHg rise in SBP, 35 (63.6 %; n=55) had a ≥ 25 mmHg rise in DBP and 55 (100 %; n=55) had a ≥ 15mmHg rise in DBP pressure. In addition, 5 (9%) women with pre-eclampsia had a SBP rise of ≥ 70mmHg and 4 (7%) had a ≥80mmHg rise. Moreover, more than half the population (56%) of women with pre-eclampsia were having a rising diastolic blood pressure of ≥ 30mmHg and one fourth of the population (27%) were experiencing an enhanced rate of ≥40mmHg. 3 (5.4%) were observed with a rise in diastolic blood pressure of ≥ 50mmHg.

Table 3.6 Rising blood pressure of women with pre-eclampsia

RISING BLOOD PRESSURE (N=55)									
SBP (mmHg)					DBP (mmHg)				
≥30	≥50	≥60	≥70	≥80	≥15	≥25	≥30	≥40	≥50
n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
52 (94.5)	28 (50.9)	12 (21.8)	5 (9.0)	4 (7.2)	55 (100)	35 (63.6)	31(56.3)	15 (27.2)	3 (5.4)

As summarized in table 3.7 the median (iqr) for gestational age at delivery was 31.2 (30.15-32.6) and 37 (36.1-38.0) for women with late on set pre-eclampsia. Mean birth weight for the women with early onset pre-eclampsia was 1.27 and 2.4 for the late onset pre-eclampsia (Table 3.7). 16 (88.8%) babies born to women with early onset pre-eclampsia were admitted to either the neonatal intensive care unit (NICU) or the prenatal baby unit (PBU). All babies born to normotensive pregnant women had a birth weight more than 2kg and were healthy.

Table 3.7 Prenatal morbidity and mortality of babies born to women with pre-eclampsia

Complications		**Early on set (≤34 weeks) (n = 18) No (%)**	**Late onset (>35weeks) (n = 37) No (%)**	**P value**	**Women with preeclampsia (n= 55) No (%)**
Gestation at delivery (weeks)	M (iqr)	31.2 (30.15-32.6)	37.0 (36.1-38.0)	-	36.0 (33.0-37.2)
Mean birth weight (kg)	(SD)	1.27 (0.40)	2.4 (0.71)	-	2.0 (0.83)
Birth weight less than 2 kg	n (%)	17 (94.0)	10 (27.0)	0.0001	27 (49.0)
Birth weight between 1-1.5kg	n (%)	14 (77.7)	3 (8.1)	0.0001	17 (30.9)
Birth weight less than 1 kg	n (%)	3 (16.6)	0	0.011	3 (5.4)
Admitted to NICU/PBU	n (%)	16 (88.8)	13 (35.1)	0.0001	29 (52.7)
Prenatal losses	n (%)	3 (16.6)	0	0.0001	3 (5.4)

Table 3.8 Phenotypic characters of women delivered pre-term at ≤ 30 weeks

	P10	**P13**	**P21**	**P26**	**P31**	**P34**
Age	26	33	38	19	33	27
BMI	29	27	26	19	18	19
BP(mmHg) at ANC	120/70	120/80	120/80	110/70	120/80	120/80
Albumin at ANC	nil	nil	nil	nil	nil	nil
Gestation at delivery (weeks)	30	30	30	30	27	29
BP(mmHg) at delivery	150/90	180/100	150/100	140/100	180/110	160/100
Albumin at delivery	3+	3+	2+	3+	3+	2+
Weight of baby at birth (kg)	1.100	1.100	1.100	0.480	0.810	1.000
Live birth	yes	yes	yes	yes	yes	yes
Survive till discharge	**Neonatal death**	yes	yes	**Neonatal death**	yes	**Neonatal death**

ANC; Antenatal Clinic, BP; Blood pressure, P10, P13, P21, P26, P31, P34 are screening number allocated for the samples in the case groups.

Table 3.8 summarizes the phenotypic characters of women with pre-eclampsia delivered pre-term at ≤ 30 weeks. 6 women with early onset pre-eclampsia developed the severe form of the disease and had no alternative but to undergo delivery via caesarean section before the 30th weeks of gestation. As illustrated in table 3.8, all babies born to the 6 women were having very low birth weights and sadly, one of them just weighed 480g. Early onset pre-eclampsia results in intra uterine growth restriction (IUGR), low fetal movement (FM) and preterm delivery. Thus, it can be stated that there's a significant risk of low birth weight babies to be born to women with pre-eclampsia and carries a higher risk of prenatal death associated with preterm delivery in women with early onset pre-eclampsia ($P = 0.0001$). 3 (5.4%) neonatal deaths were recorded from the case group (women with pre-eclampsia). The phenotypic data obtained from the study, further emphasises and confirms pre-eclampsia is associated with severe maternal and prenatal morbidity and mortality.

Table 3.9 summarizes the phenotypic data of women with pre-eclampsia recruited to the current study (2010-2011) with that of a similar cohort recruited in 2001-2003.

Table 3.9 Comparison of data between the periods 2001-2003 and 2010-2011

Complications		During 2010-2011 n=55; n (%)	During 2001-2003 n=180; n (%)
SBP ≥ 160mmHg		30 (54.5)	136 (75.5)
DBP ≥ 110mmHg		17 (30.9)	151 (83.8)
Proteinuria (≥3+HCT)		28 (50.9)	156 (86.6)
Renal impairment requiring dialysis		2 (3.6)	4 (2.2)
Thrombocytopenia (<100x10^9 /L)		5 (9.0)	23 (12.7)
Elevated liver enzyme (AST and/or ALT ≥ 70 U/L)		5 (9.0)	19 (10.5)
Developed eclampsia		8 (14.5)	16 (8.8)
Admitted to Intensive care unit		13 (23.6)	No data
Maternal death		0	1 (1.3)
Mean birth weight (kg) (SD)	Early onset PE	1.27 (0.40)	1.13 (0.48)
	Late onset PE	2.4 (0.71)	2.32 (0.58)
Birth weight below 2 kg		27 (49.0)	No data
Birth weight below 1 kg		3 (5.4)	No data
Admitted to NICU/PBU		29 (52.7)	No data
Perinatal losses		3 (5.4)	45 (25)

SBP, systolic blood pressure; DBP, diastolic blood pressure; AST, aspartate aminotransaminase; ALT, alanine aminotransaminase; HCT, the urine protein heat coagulation test.

RESULTS- PHASE 1

3.2 The Metagenomics Analysis

3.2.1 Results of bacterial metagenomics- Placental tissue samples

As illustrated in figure 3.3 a 500bp band on the agarose gel indicates the presence of 16S rRNA gene, giving positive results for the presence of bacteria.

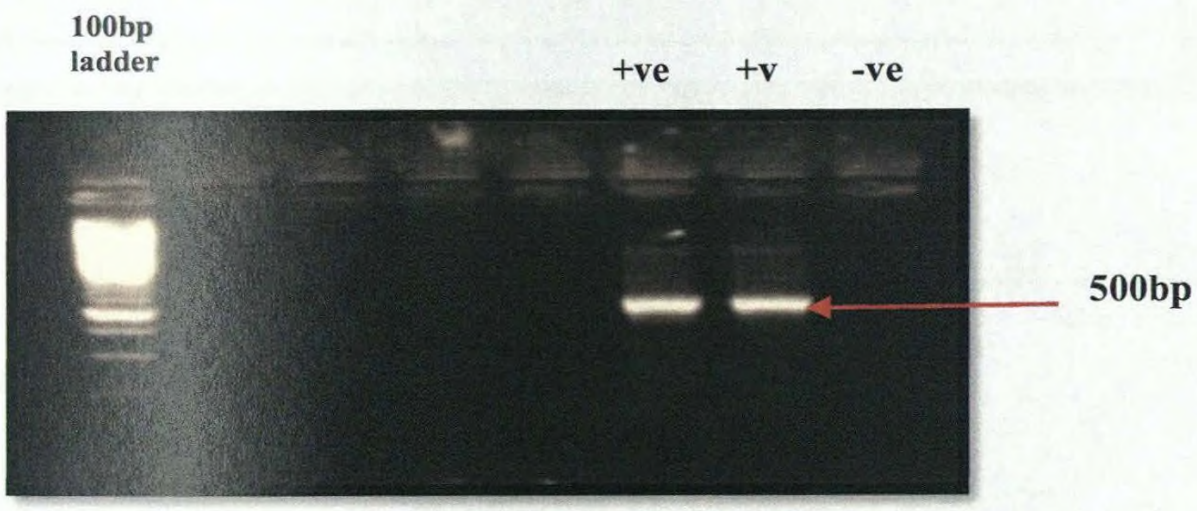


Figure 3.3 Gel picture of 16S rRNA PCR assay

500bp band on the agarose gel indicates the presence of 16S rRNA gene, giving positive results for the presence of bacteria.

16S rRNA PCR test carried out to detect the presence of bacteria in placental tissue samples obtained from women with pre-eclampsia resulted in 7 (12.7%) positives. The 16S ribosomal RNA was detected in 7 samples and gave a 500bp band (as illustrated in figure 3.3) when visualized on an agarose gel. None of the control group (normotensive women) was positive for the presence of bacteria. The 7 positive samples were then sequenced using Sanger sequencing method. Figure 3.4 is an electrophorogram with clear peaks indicates a presence of a single bacterium and facilitate easy identification of the bacterium present. Multiple overlapping nucleotide peaks in the electrophorogram as in figure 3.5 indicates the presence of

mixed infection. Accordingly, 6 (85.7%) of the seven positive samples were indicating the presence of mixed infection.

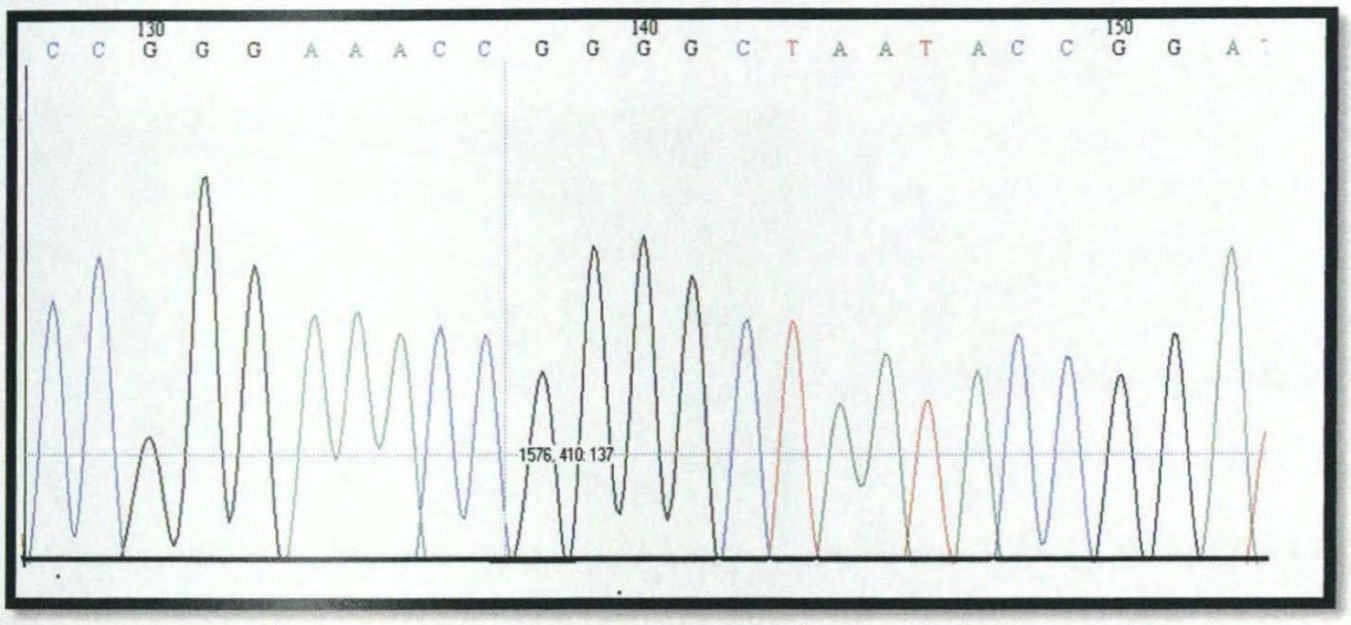


Figure 3.4 Clear Electrophorogram

Electrophorogram with clear peaks indicates a presence of single bacterium and facilitate easy identification of the bacterium present.

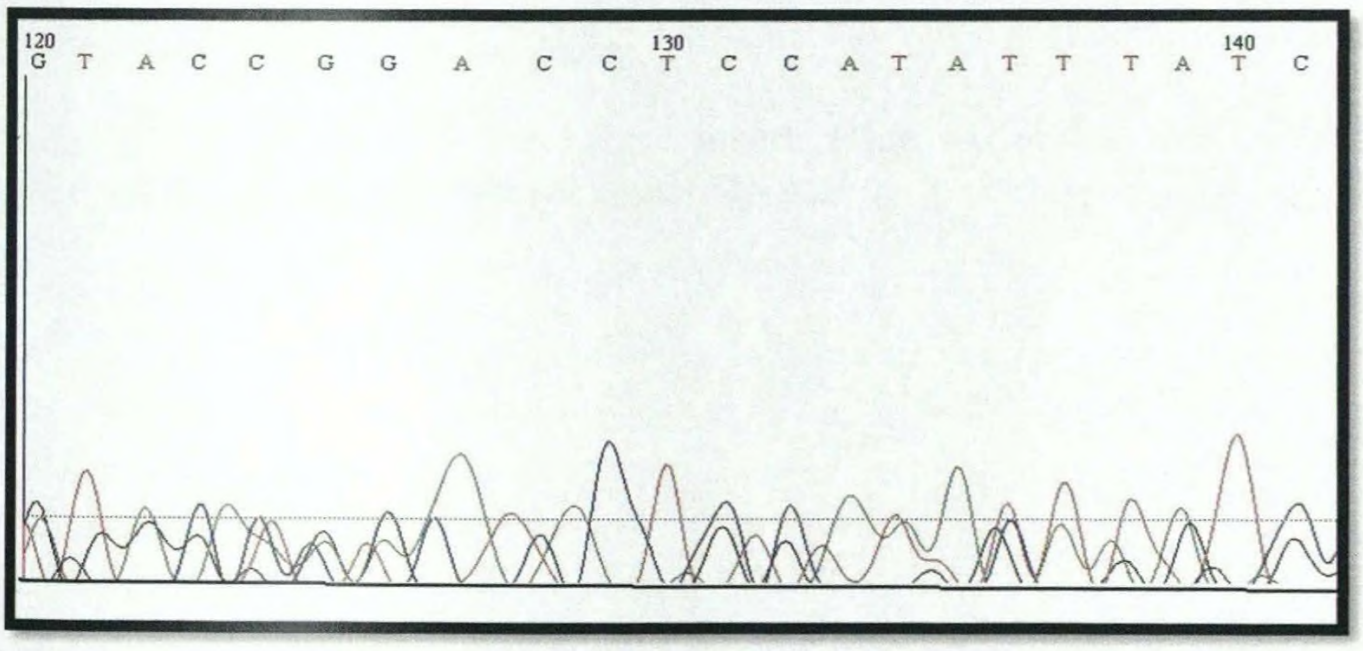


Figure 3.5 Distorted Electrophorogram

Multiple overlapping nucleotide peaks in the electrophorogram indicates the presence of mixed infection.

The 16S rRNA gene of the samples which had mixed infections was cloned in to pGEM-T and was transformed in to competent JM109 cells. White colonies are colonies with the 16S rRNA gene of interest as shown in figure 3.6 below.

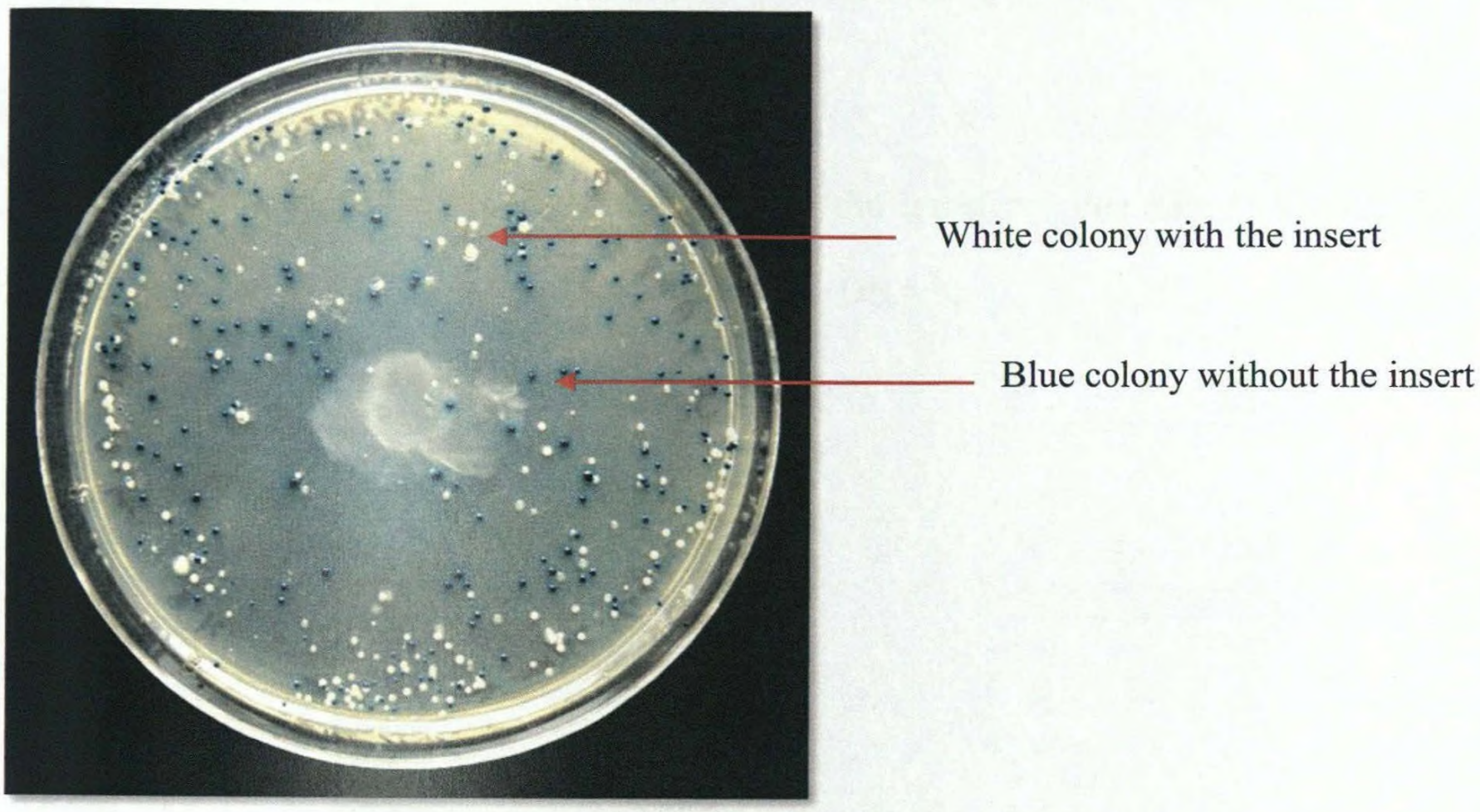


Figure 3.6 'Blue and White' colony plate

White colonies are colonies with the target insert. Blue and white colony plate helps in identifying the colony carrying the target insert DNA.

Transformation Efficiency was calculated as below:

$$\text{Transformation efficiency} = \frac{\text{Number of colony forming units}}{0.001\text{ng}}$$

$$\text{Average colony forming units} = \frac{250+170}{2} = 210 \text{ cfu}$$

$$\text{Transformation efficiency} = \frac{210\text{cfu}}{0.001\text{ng}} = 2\text{x}10^5 \text{ cfu/ng} = 2\text{x}10^8 \text{ cfu/}\mu\text{g DNA}$$

Accordingly, the JM109 competent cells used for the transformation step in the cloning experiment was having a competency of $2\text{x}10^8$cfu/µg DNA.

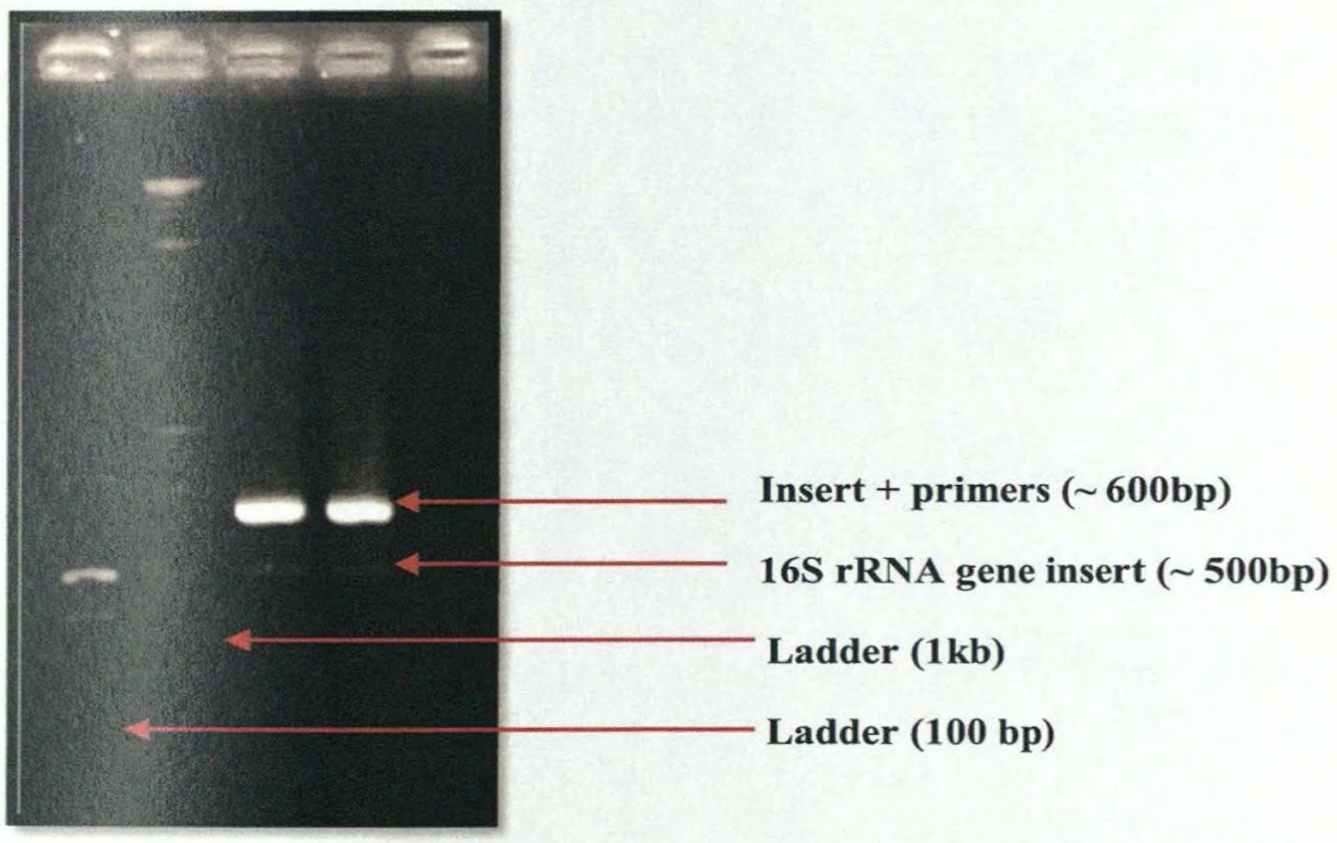


Figure 3.7 Gel picture of the colony PCR product

Band at 500bp indicates the plasmid prepared contains the insert of interest (500bp). Band at 600bp indicates the insert amplified with the T7 and SP6 primers. This gel suggests a successful cloning of the insert DNA.

The gel picture in figure 3.7 illustrates result of a colony PCR performed to confirm the presence of 16S rRNA gene of insert in the white colonies selected.

The samples confirmed by colony PCR was Sanger sequenced and the following results were obtained. The bacteria identified from women with pre-eclampsia were *Bacillus cereus* in 1 sample (14.2%); *Bacillus circulans* in 1 sample (14.2%); *Klebsiella pneumonia* in 2 samples (28.5%); Lactobacillus iners in 1 sample (14.2%); *Bacillus sp.* in 4 samples (57.1%); *Firmicutes* in 6 sample (85.7%); *Proteobacterium* in 1 sample (14.2%); Uncultured bacteria in 3 samples (42.8%). Multiple infections were found in 6 (85.7%) samples.

Table 3.10 Bacteria identified from the placental tissues of women with pre-eclampsia

Women with Preeclampsia Screening number	Results Bacteria Identified
P9	▪ *Bacillus cereus*
P23	▪ *Bacillus* species ▪ *Stenotrophomonas* ▪ *Pseudoxanthomonas*
P26	▪ *Klebsiella pneumoniae* ▪ *Proteobacterium* ▪ Uncultured bacteria
P30	▪ *Bacillus* species ▪ *Bacillus circulans* ▪ *Bacillus thermoamylovorans* ▪ *Anoxybacillus flavithermas* ▪ *Klebsiella pneumoniae*
P34	▪ Uncultured bacteria*
P36	▪ *Bacillus* species ▪ *Anoxybacillus flavithermus*
P54	▪ Uncultured bacteria ▪ Fermicutes ▪ *Lactobacillus iners*

*Sample number P34 failed to identify the bacteria up to genus level. Refer annexures 7.1 for the BLAST reports of the organisms identified.

3.2.2 Results of fungal metagenomics- Placental tissue samples

Only 1 (1.8%) sample, patient screening no P5, was detected positive for the presence of fungi in women with pre-eclampsia. However, none of the normotensive women was detected positive for the presence of fungi. The fungi identified using fungal metagenomics was '*Malassezia restricta*' strain. None of the normotensive women were positive for the presence of fungi.

3.2.3 Results of viral metagenomics- Placental tissue samples

None of the samples, from both cases and controls were detected positive for the presence of viruses.

3.2.4 Results of bacterial/ fungal metagenomics- Amniotic fluid samples

1 (2.0%) sample out of the 48 amniotic fluid samples of women with pre-eclampsia was detected positive for the presence of bacteria. However, there were no fungi detected in women with pre-eclampsia. In addition, none of the normotensive women were detected positive for the presence of bacteria or fungi. The sample detected positive for the presence of bacteria in women with pre-eclampsia was the amniotic fluid sample with the screening number P9 and the bacteria was identified as *Bacillus cereus*.

3.2.5 Results of bacterial/fungal metagenomics- Blood samples

3 (5.5%) samples from the women with pre-eclampsia were positive for the presence of bacteria but none were detected positive for fungi. Similar to the previous results, none of the blood samples obtained from normotensive women were detected positive for the presence of bacteria or fungi. The bacteria identified in the blood samples of women with pre-eclampsia is illustrated in Table 3.11 below.

Table 3.11 Bacteria identified from the blood samples of women with pre-eclampsia

Women with Preeclampsia **Screening number**	**Results** **Bacteria Identified**
P7	▪ *Reyranella* sp. ▪ Uncultured proteobacterium
P8	▪ *Reyranella* sp.
P10	▪ *Ralstonia picketti*

3.2.6 Results of bacteria/fungi metagenomics- Urine samples

None of the case group nor the control group women were detected positive for the presence of fungi. However, 8 (14.5%) women with pre-eclampsia and 9 (16%) normotensive pregnant women were detected positive for the presence of bacteria. Table 3.12 summarizes the bacteria identified from the urine samples of these women.

Table 3.12 Bacteria identified from the urine samples

Women with pre-eclampsia	Bacteria identified	Normotensive women	Bacteria identified
P3	*Lactobacillus gasseri*	**N3**	Uncultured bacteria
P11	Uncultured bacteria	**N21**	Uncultured bacteria
P13	Uncultured bacteria	**N24**	*Prevotella bivia*
P18	*Veillonella montpellierensis*	**N33**	Uncultured bacteria
P21	*Lactobacillus criptus*	**N37**	Uncultured bacteria
P37	*Veillonella montpellierensis*	**N42**	*Bacillus sp.*
P46	*Veillonella montpellierensis*	**N48**	*Lactobacillus sp.*
P50	Uncultured bacteria	**N50**	*Atopobium vaginae*
		N54	*Streptococcus agalactiae*

3.2.7 Phase 1 results in summary

At the end of phase 1 of the current study, placenta of 7 (12.7%) of 55 women with pre-eclampsia were positive for bacteria. Figures 3.8, 3.9, 3.10, 3.11, 3.12 illustrate the phylogenic trees of bacteria identified in the placental tissue samples of women with pre-eclampsia. 1 (1.8%) was positive for fungi and none (0%) were positive for viruses. None (0%) of the normotensive pregnant women samples were positive for the presence of bacteria, fungi or viruses.

Other biological samples obtained from these placenta during delivery via caesarean section were further tested using 16S/28S metagenomics for the detection of bacteria and fungi. 1(2%) of the 48 amniotic fluid samples of women with pre-eclampsia were positive for bacteria but none were positive for fungi. None (0%) of the normotensive women samples were positive for the presence of bacteria, fungi or viruses. 3 (5.5%) of the 54 blood samples of women with pre-eclampsia were positive for bacteria but fungi were not detected. None (0%) of the normotensive women blood samples were positive for bacteria, fungi or viruses. 8 (14.5%) of the urine samples obtained from women with pre-eclampsia and 9 (16%) of the urine samples obtained from normotensive women were positive for bacteria. However, none of the cases or control samples was positive for fungi.

The table 3.13 illustrates the bacteria identified from the biological samples of women with pre-eclampsia and normotensive pregnant women. Accordingly, even though amniotic fluid, blood, urine gave preliminary evidence for the presence of bacteria, only the bacteria present in placental tissues were statistically significant (P=0.006). In view of the above, it can be safely concluded that at the end of phase 1, preliminary evidence is available on the presence

of bacteria in the placental tissues of women with pre-eclampsia. Placental bacteria may play a significant role in the multifactorial aetiology of pre-eclampsia. This is further analysed by novel metagenomics using next generation sequencing in phase 2 of the study. Table 3.14, illustrates the fungi identified from the biological samples of women with pre-eclampsia. Only 1 sample was positive from the placental tissue samples and was identified as *Malassezia restricta* strain, which can be ignored as it is considered a normal commensal on skin. This will be discussed in detail in chapter 4, Discussion. Table 3.15, summarizes the bacteria identified in the biological samples of women with pre-eclampsia and normotensive women.

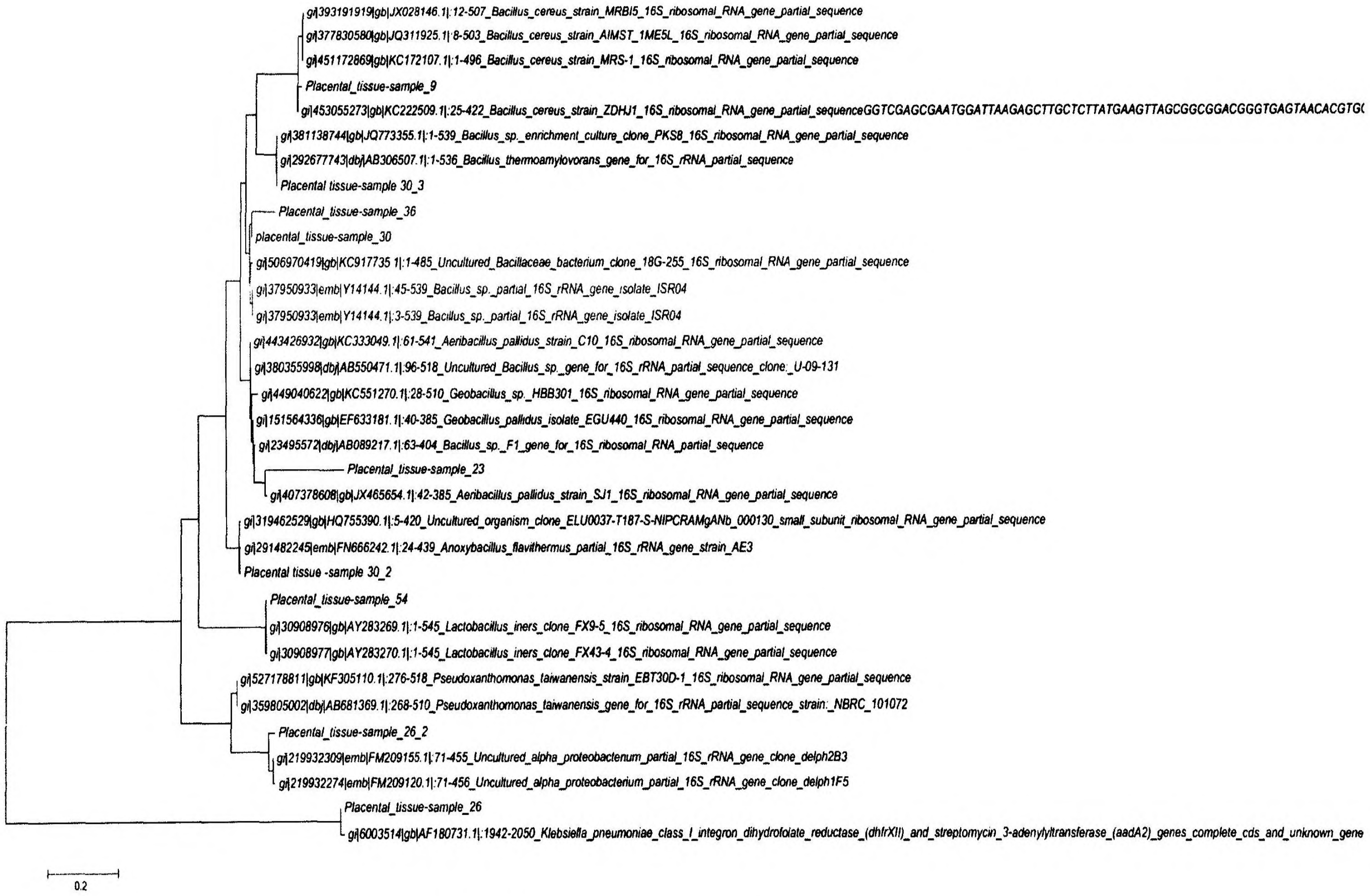


Figure 3.8 Phylogenic tree of bacteria identified from placental tissue samples

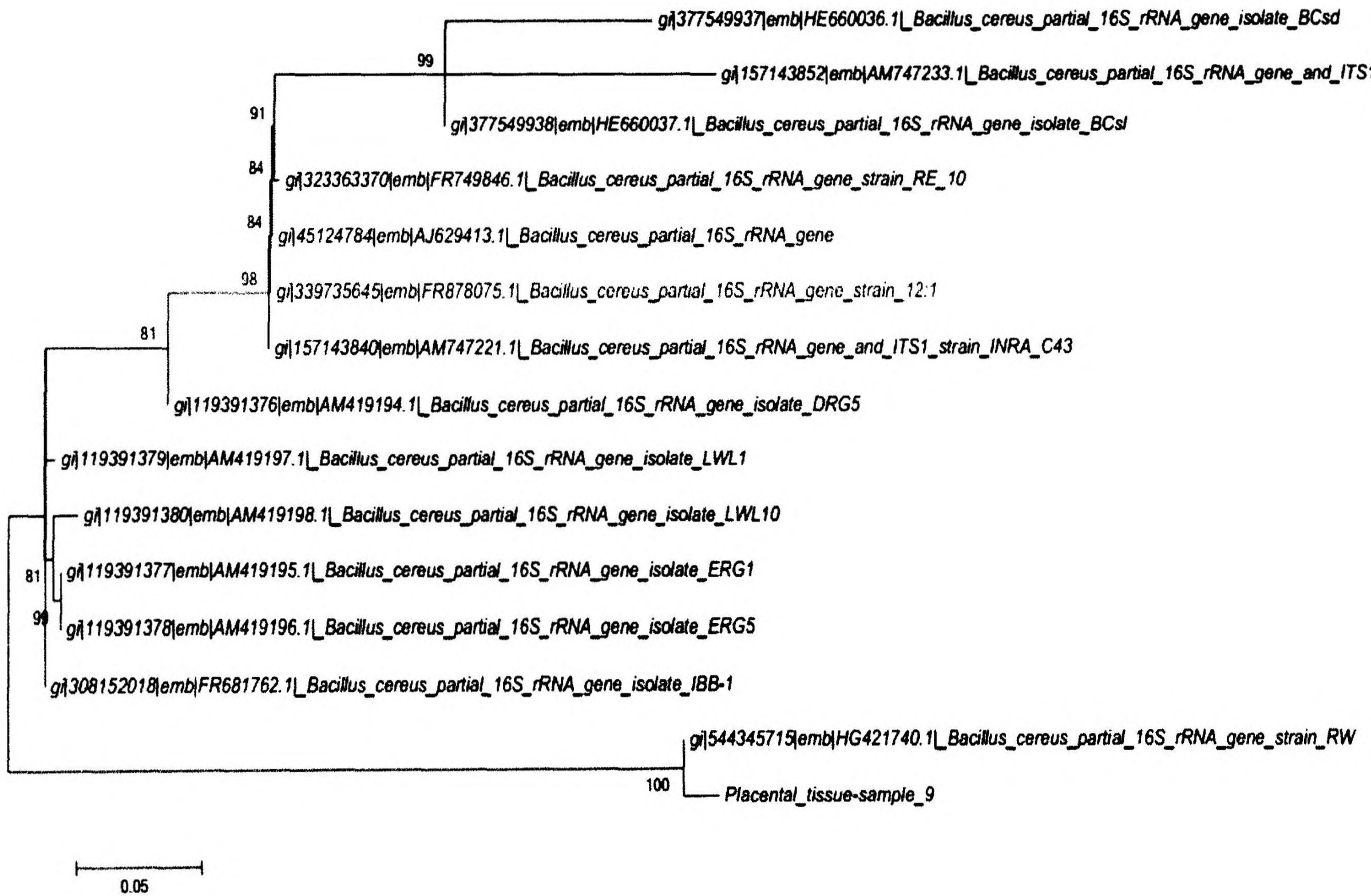


Figure 3.9 Phylogenic tree for *Bacillus cereus* identified in sample P9

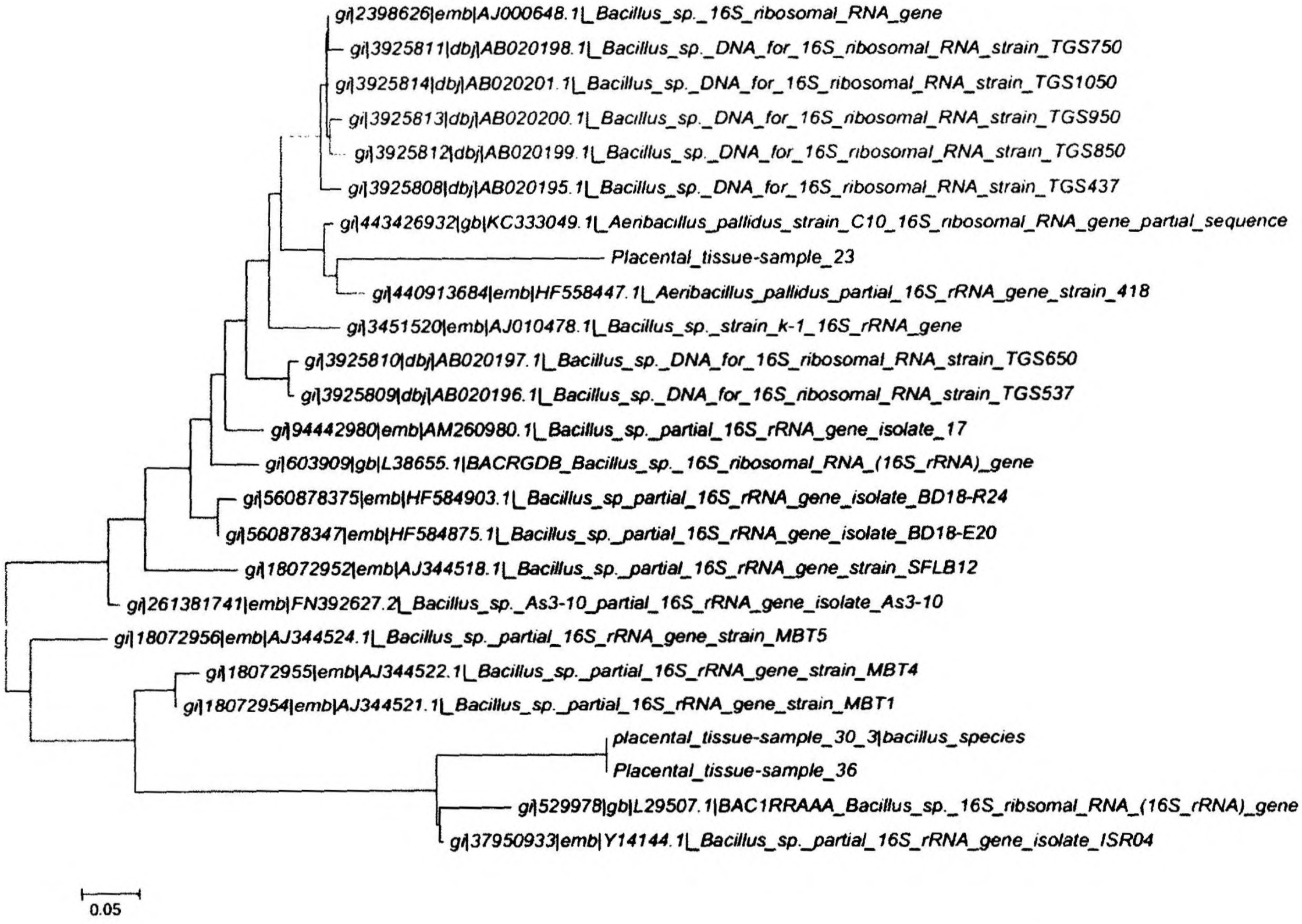


Figure 3.10 Phylogenic tree of *Bacillus sp.* identified in samples P23, P30 & P36

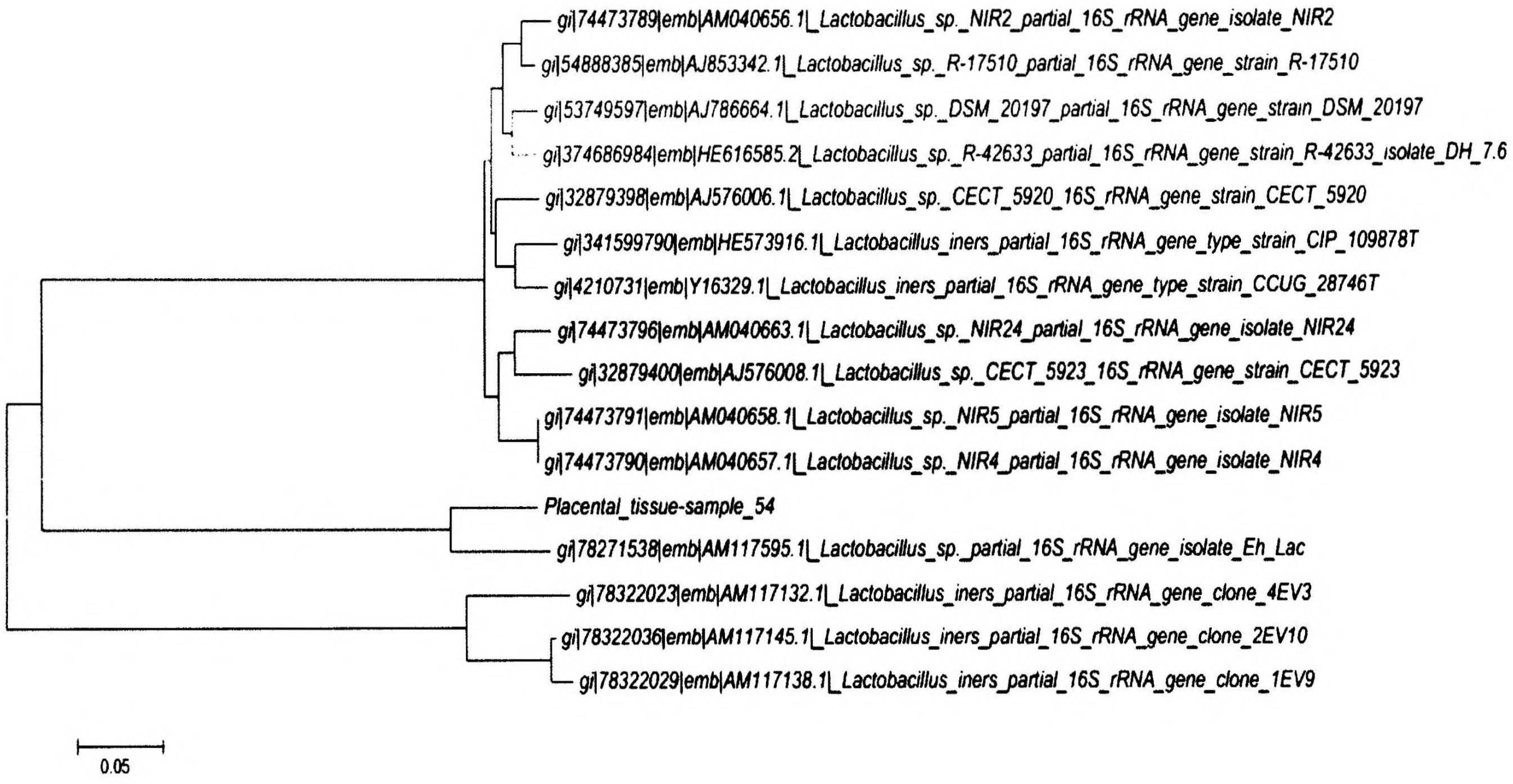


Figure 3.11 Phylogenic tree of *Lactobacillus iners* identified in sample 54

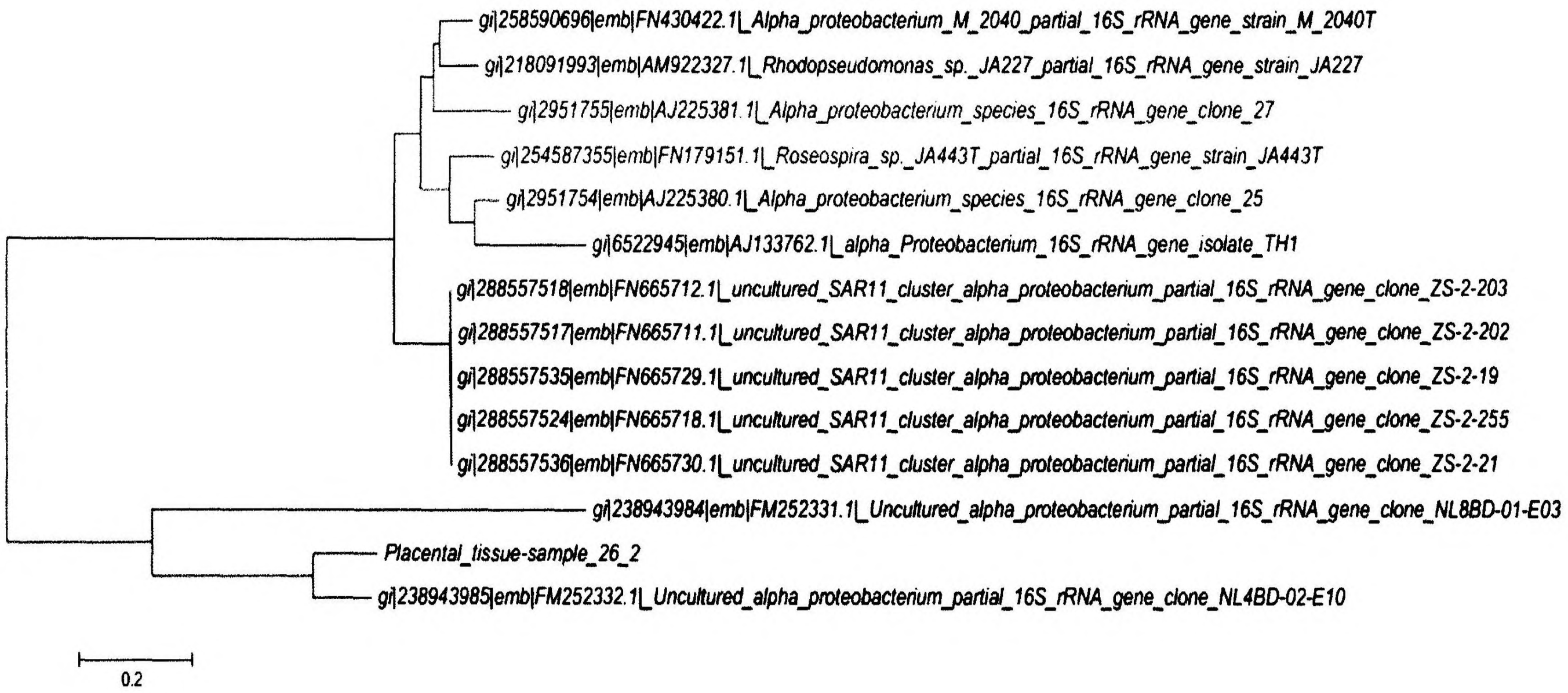


Figure 3.12 Phylogenic tree of Proteobacterium identified in sample P26

Table 3.13 Biological samples tested positive for the presence of bacteria

Sample type	Women with Pre-eclampsia (Cases)			Normotensive Women (Controls)			P value
	Total no of sample tested	No of positive samples	%	Total no of sample tested	No of positive samples	%	
Placental tissues	55	7	12.7	55	0	-	**0.006**
Amniotic Fluid	48	1	2.0	48	0	-	0.332
Blood	54	3	5.5	54	0	-	0.090
Urine	55	8	14.5	55	9	16.0	0.960

Table 3.14 Biological samples tested positive for the presence of fungi

Sample type	Women with Pre-eclampsia (Cases)			Normotensive Women (Controls)		
	Total no of sample tested	No of positive samples	%	Total no of sample tested	No of positive samples	%
Placental tissues	55	1	1.8	55	0	-
Amniotic Fluid	48	0	-	48	0	-
Blood	54	0	-	54	0	-
Urine	55	0	-	55	0	-

Table 3.15 Bacteria identified in the biological samples

Sample type	Bacteria detected	Diseases caused by the specific bacteria
Placental tissue (cases)	*Bacillus cereus*	Gastroenteritis
	Bacillus circulans	Respiratory tract infection
	Bacillus	Respiratory tract infection
	Klebsiella pneumoniae	Respiratory tract infection
	Fermicutes	Respiratory tract infection
	Proteobacterium	Gastroenteritis, Respiratory tract infection
Amniotic Fluid (Cases)	*Bacillus cereus*	Gastroenteritis
Blood (Cases)	*Reyranella* sp	Non Pathogenic
	Ralstonia picketti	Respiratory tract infection
Urine (Cases)	*Lactobacillus gasseri*	Normal flora
	Lactobacillus crispatus	Normal flora
	Veillonella montpellierensis	Normal flora
Urine (Controls)	*Prevotella bivia*	Normal flora
	Atopobium vaginae	Normal flora
	Streptococcus agalactiae	Normal flora
	Lactobacillus sp.	Normal flora

***fungi detected in the placental tissue of women with pre-eclampsia was *Malassezia restricta*, a known commensal**

RESULTS-PHASE 2

3.3 The Metagenomics Analysis

3.3.1 Bacterial identification using 16S Metagenomics NGS

3.3.1.1 Results of whole gene amplification of 16S rRNA gene

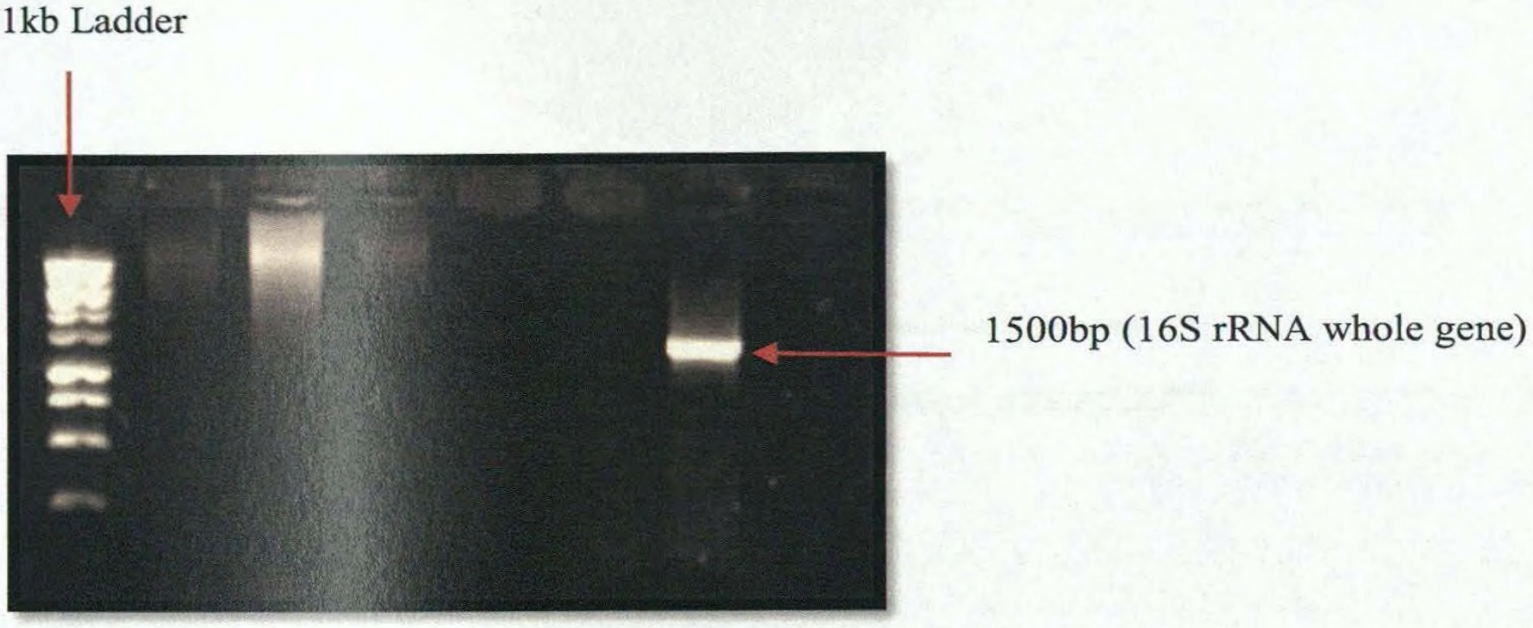


Figure 3.13 Gel picture visualizing the whole genome of 16S rRNA gene

Band at 1500bp indicates the whole genome amplification of the 16S rRNA gene of bacteria.

The amplification of the whole genome of the 16S ribosomal RNA gene gave a 1500bp band when run on a 1.5% agarose gel as indicated in Figure 3.13. Seven (12.7%) placental tissue samples which became positive with the 16S partial gene amplification were re-detected for the presence of bacteria using the 16S whole genome amplification. None in the control group (normotensive women) resulted in a positive even with the whole genome amplification. Accordingly, the 7 samples which was analysed using Sanger sequencing were further analysed using the 16S metagenomics next generation sequencing technology.

3.3.1.2 Metagenomics results by 'Illumina MiSeq Reporter'

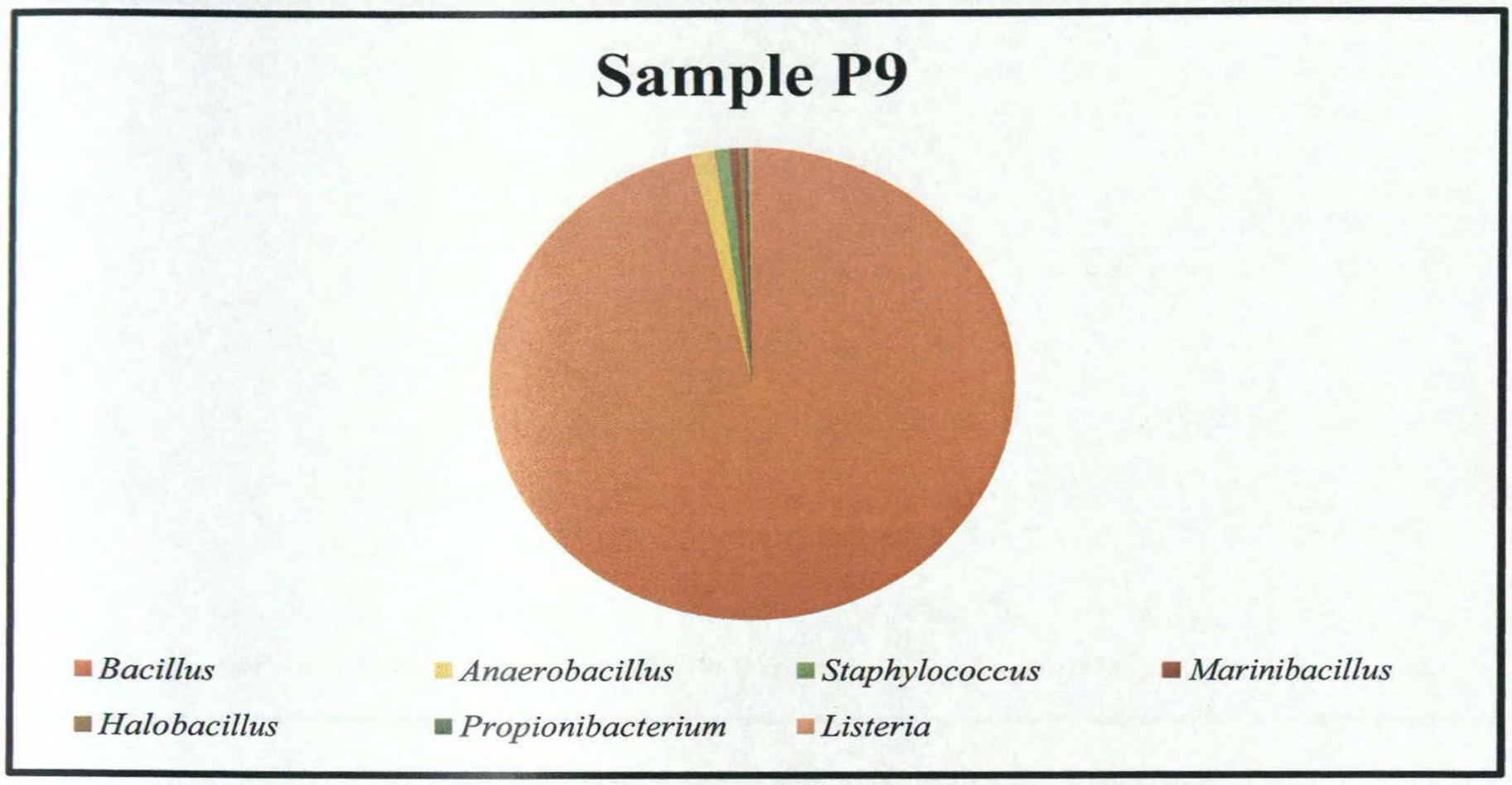


Figure 3.14 Genus level classification of sample P9

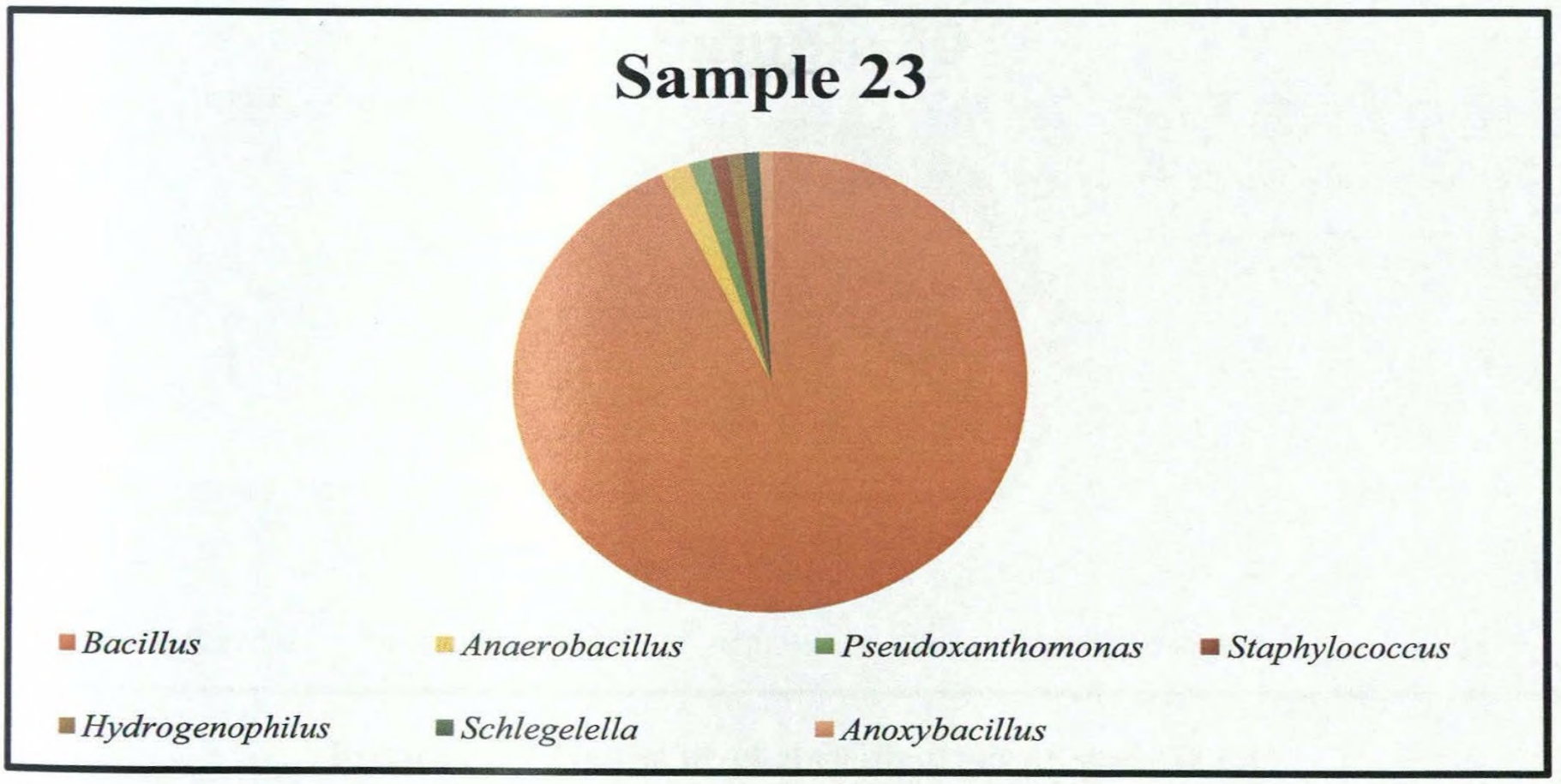


Figure 3.15 Genus level classification of sample P23

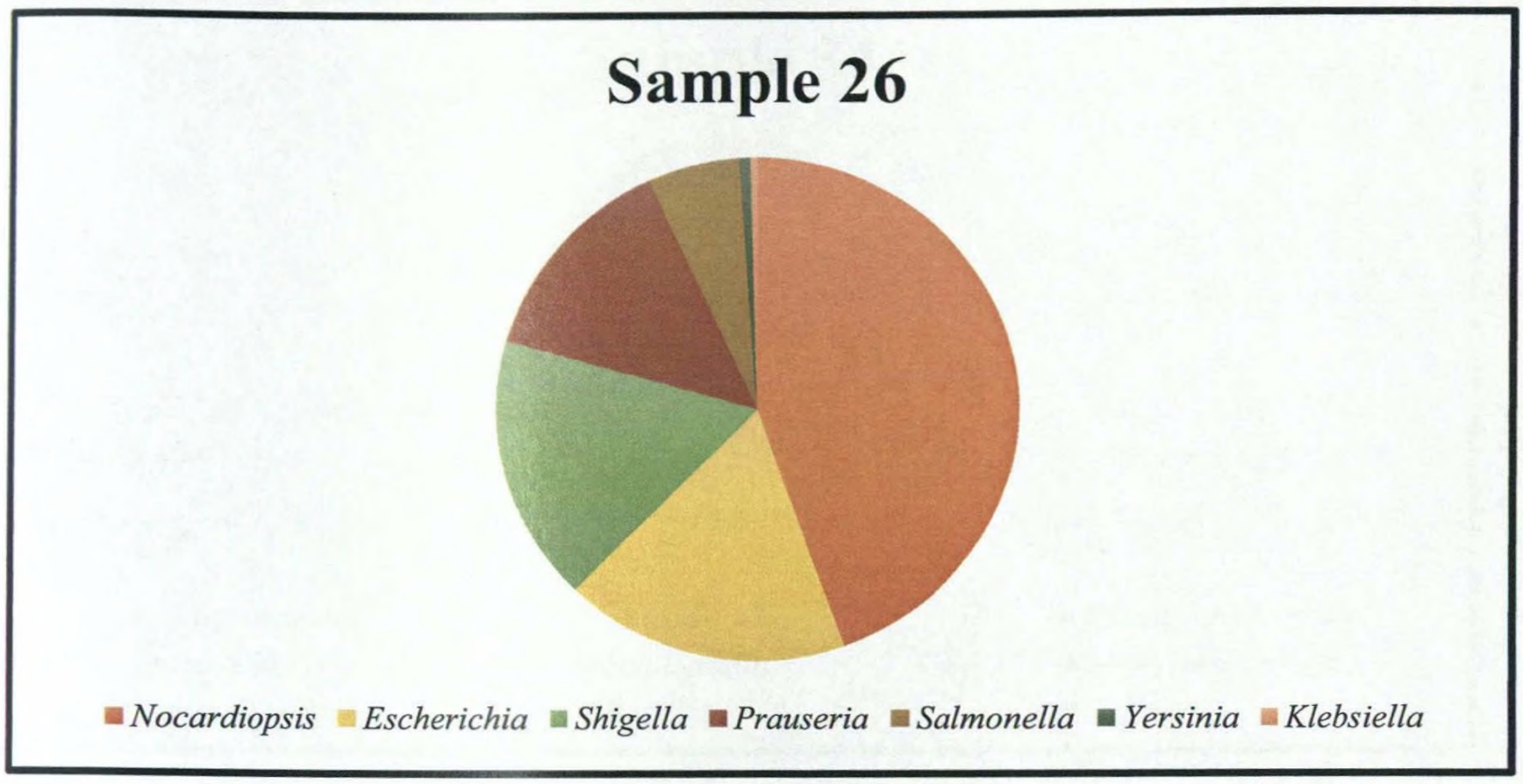


Figure 3.16 Genus level classification of sample P26

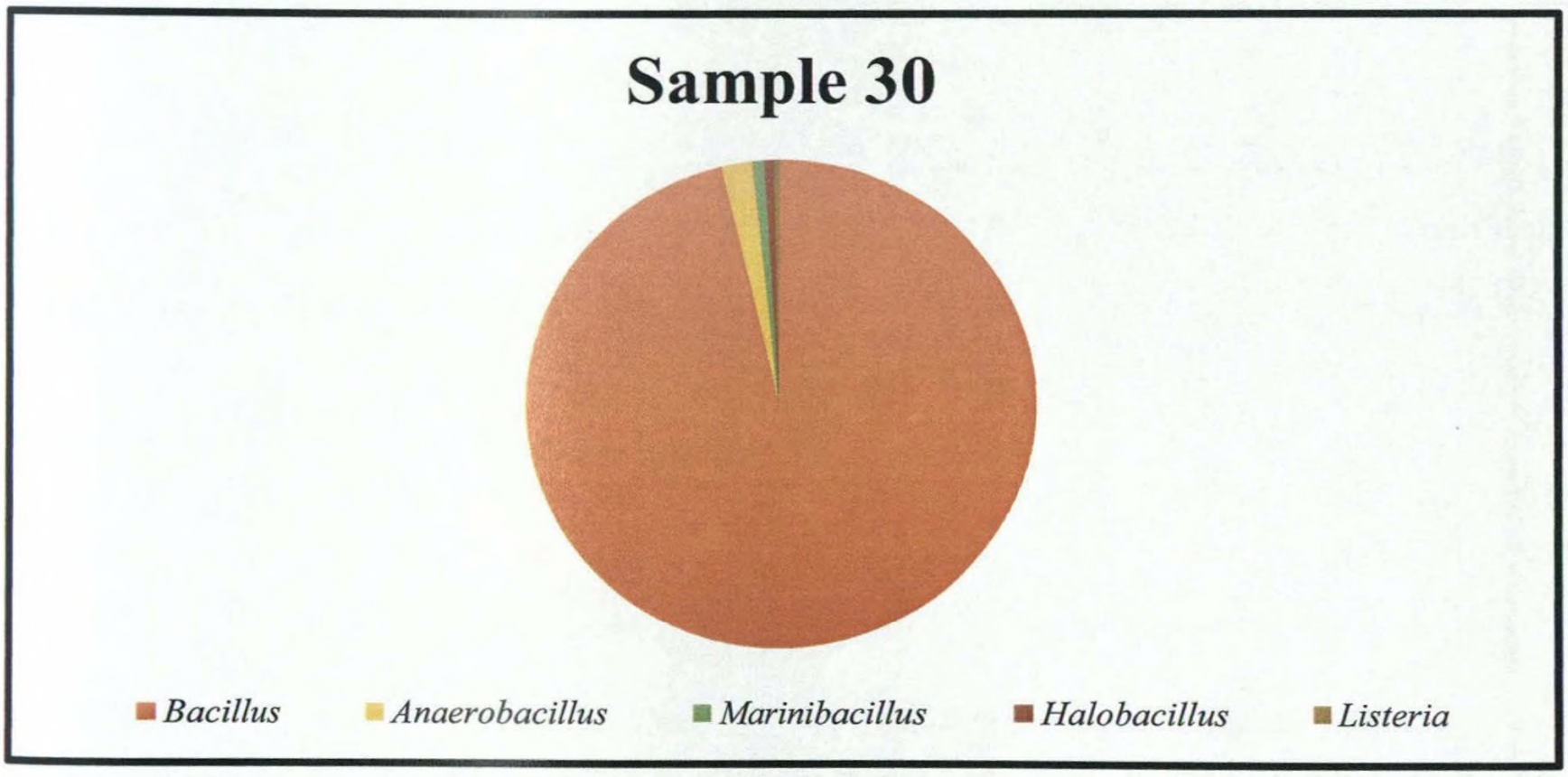


Figure 3.17 Genus level classification of sample P30

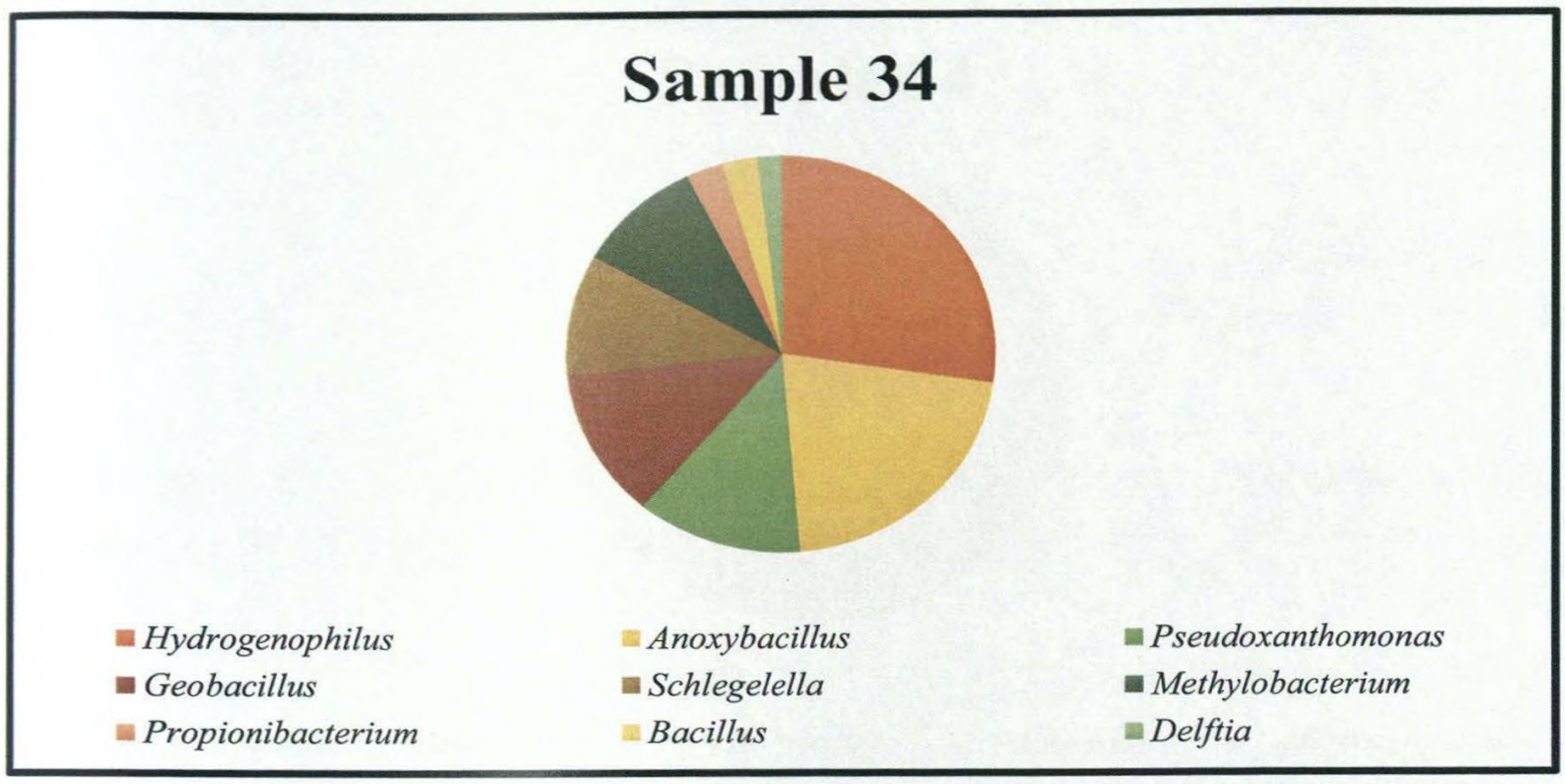


Figure 3.18 Genus level classification of sample P34

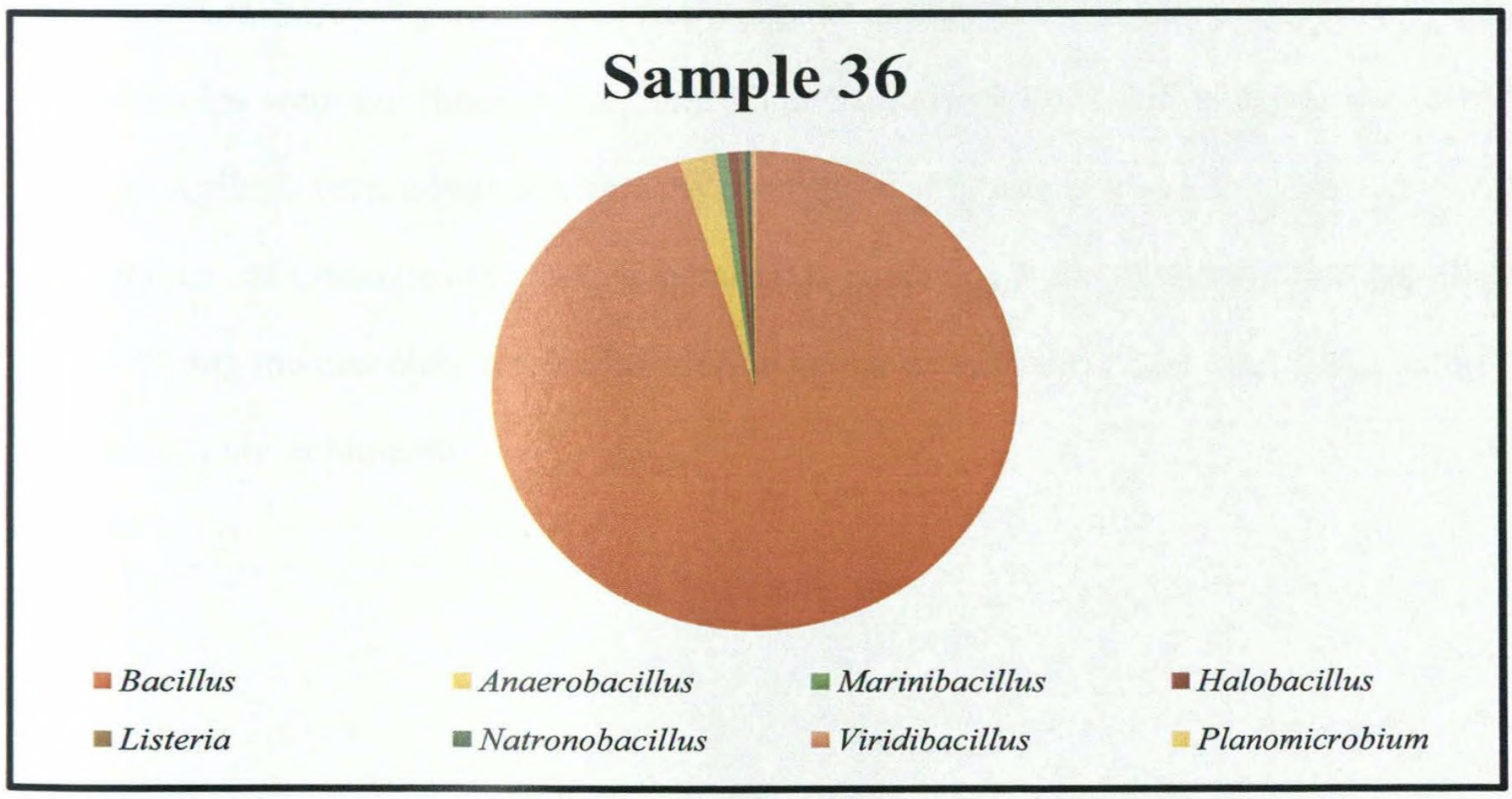


Figure 3.19 Genus level classification of sample P36

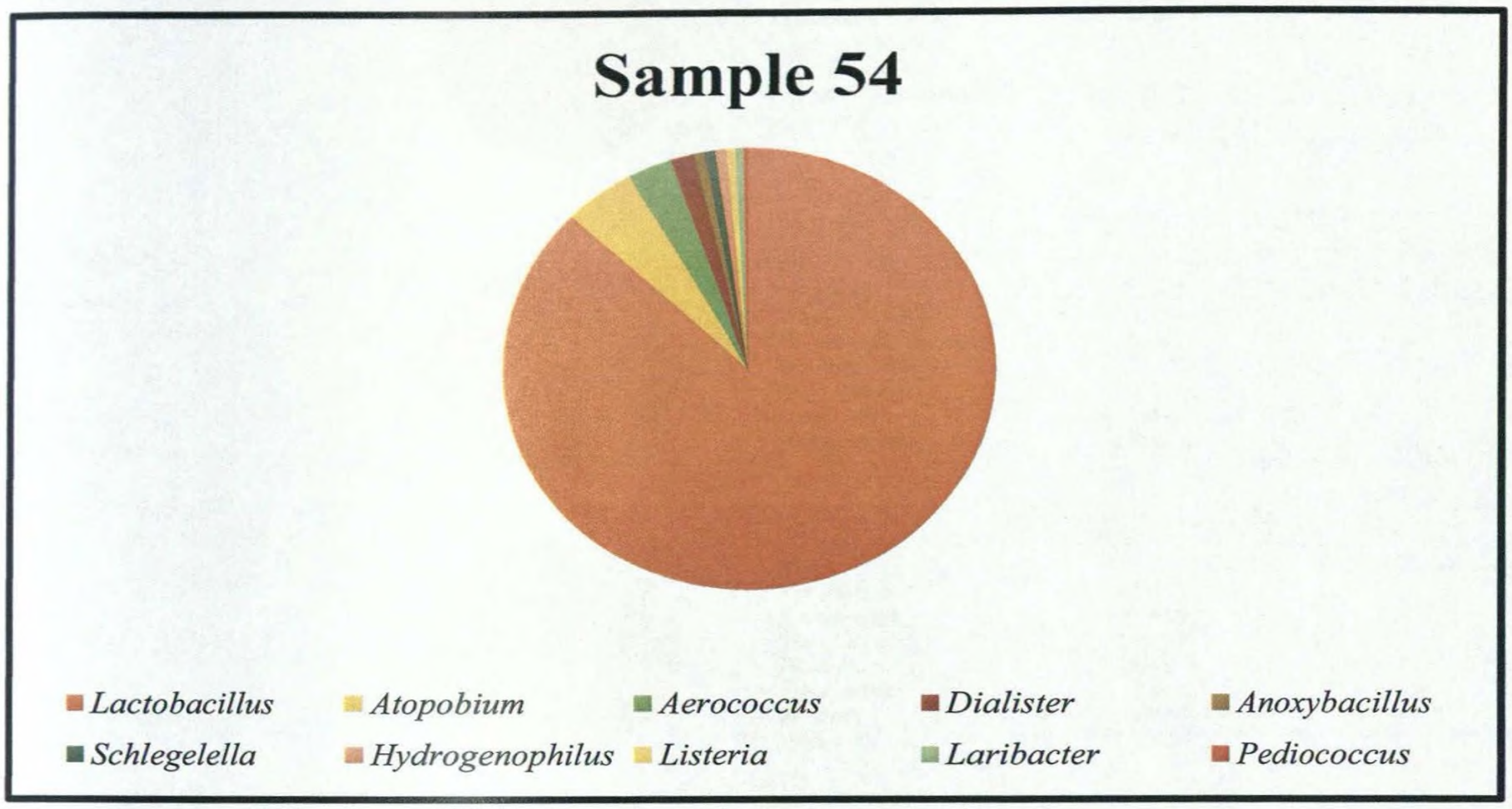
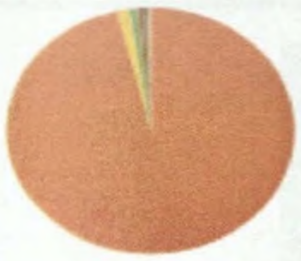


Figure 3.20 Genus level classification of sample P54

Prior to performing next generation sequencing, the quality of the library pool of the 7 placental tissue samples were confimed by Agilent High Sensitivity DNA kit as explained in section 2.14.4.8 (Agilent Technologies, Germany) and the test report is given in annexures 7.2. The above pie charts illustrates the next generation sequening results obtained using the Illumina MiSeq, giving the complete microbiome up to genus level of the 7 placental tissue samples of women with pre-eclampsia.

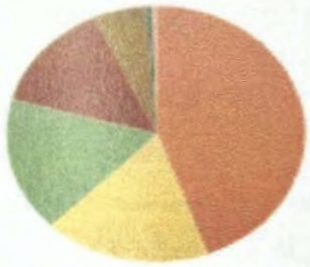


P9

Bacillus	96.19%
Anaerobacillus	1.49%
Staphylococcus	0.87%
Marinibacillus	0.57%
Halobacillus	0.42%
Propionibacterium	0.24%
Listeria	0.23%

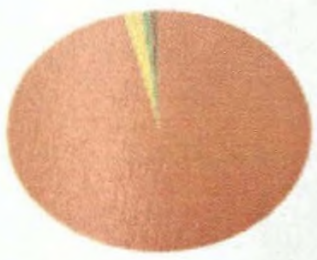
P23

Bacillus	92.91%
Anaerobacillus	1.86%
Pseudoxanthomonas	1.29%
Staphylococcus	1.10%
Hydrogenophilus	1.04%
Shlegelella	0.98%
Anoxybacillus	0.82%



P26

Nocardiopsis	44.46%
Escherichia	17.91%
Shigella	17.05%
Praseria	13.69%
Salmonella	5.77%
Yersinia	0.71%
Klebsiella	0.41%

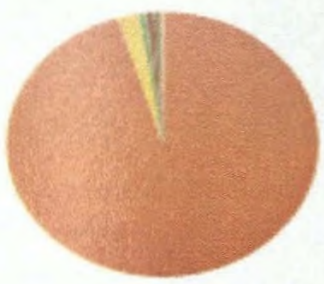


P30

Bacillus	96.17%
Anaerobacillus	2.01%
Marinibacillus	0.71%
Halobacillus	0.67%
Listeria	0.43%

P34

Hydrogenophilus	27.38%
Anoxybacillus	21.24%
Pseudoxanthomonas	12.62%
Geobacillus	9.94%
Shlegella	9.52%
Methylobacterium	2.71%
Bacillus	2.68%
Delfia	1.83%



P36

Bacillus	95.52%
Anaerobacillus	2.29%
Marinibacillus	0.73%
Halobacillus	0.68%
Listeria	0.45%
Natronobacillus	0.32%
Varidibacillus	0.21%
Planomicrobium	0.11%

P54

Lactobacillus	87.27%
Aatopobium	4.72%
Aerococcus	2.82%
Dialister	1.57%
Anoxybacillus	0.73%
Schlegella	0.72%
Hydrogenophilus	0.72%
Listeria	0.67%
Laribacter	0.51%
Pediococcus	0.26%

Figure 3.21 Genus level classification of bacteria with percentage

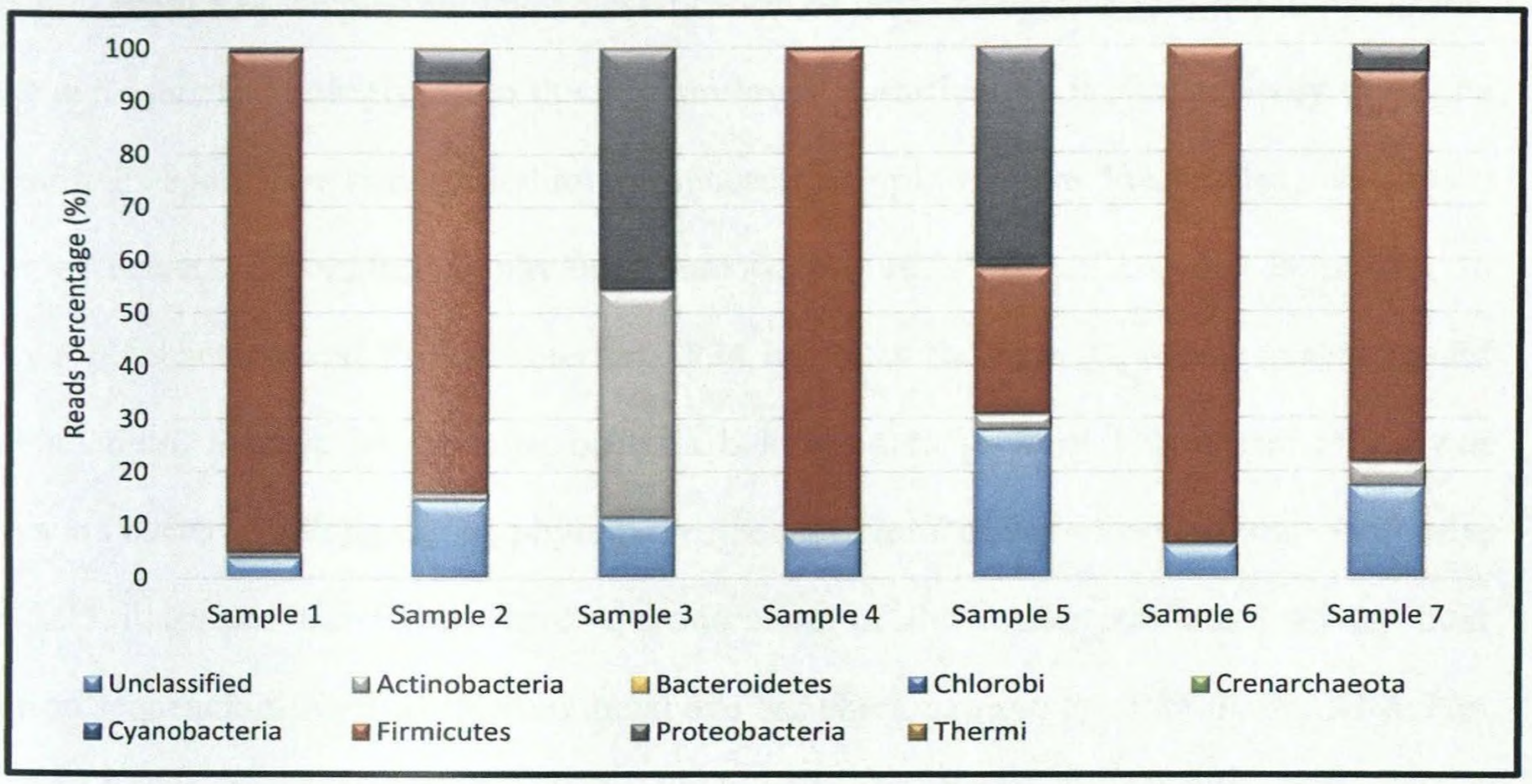


Figure 3.22 Phylum level classification

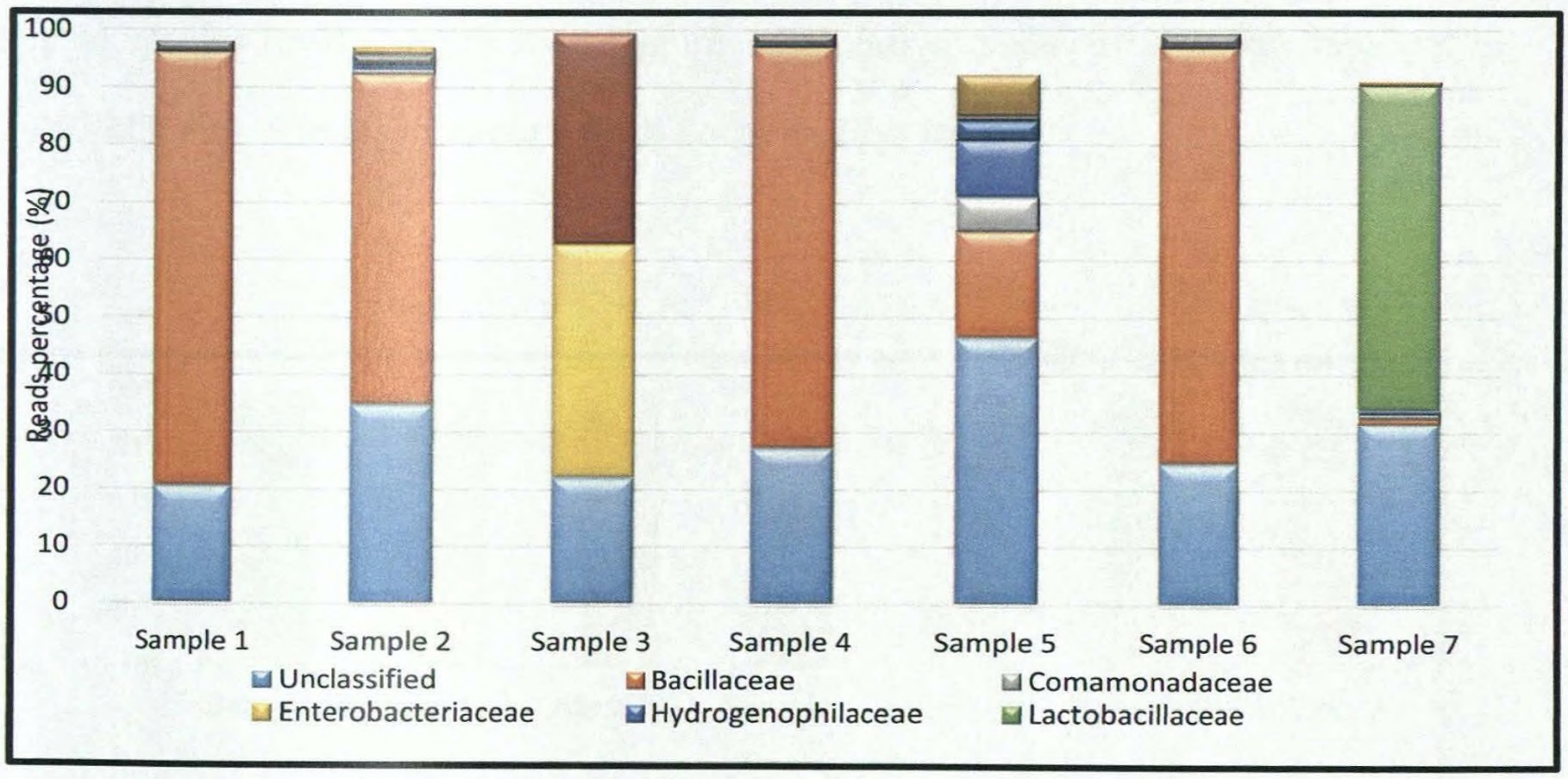


FIgure 3.23 Family level classification

Figure 3.22 illustrates the phylum level classification of the 7 samples according to the MiSeq reporter analysis. Accordingly, from the phylum level classification it can be easily observed that sample P9 and P36 is rich in Phylum Fermicutes. Sample P23 has Fermicutes and a small number of bacteria belonging to phylum Proteobacterium. P26 has bacteria belonging to phylum Actinobacteria and Proteobacterium. P34 has bacteria from phylum Fermicutes and Proteobacterium. Sample 54 contains bacteria belonging to phylum Fermicutes and fewer numbers of bacteria belonging to phylum Proteobacterium and Actinobacteria. Similarly, figure 3.23 illustrates the family level classification of the 7 samples analysed by next generation sequencing. As illustrated in the above bar chart, sample P9, P23, P30, P34 & P36 contains bacteria belonging to the family Bacillaceae. Sample P26 contains Enterobacteriaceae and Nocardiopsaceae whilst the sample P54 contains Lactobacillus. Apart from these, sample no P23 contains fewer numbers of bacteria belonging to the family Enterobacteriaceae. According to species level classification (figure 3.24) *Bacillus cereus* is more abundant in samples P9, P23, P30, P36 and sample 54 has *Lactobacillus iners*.

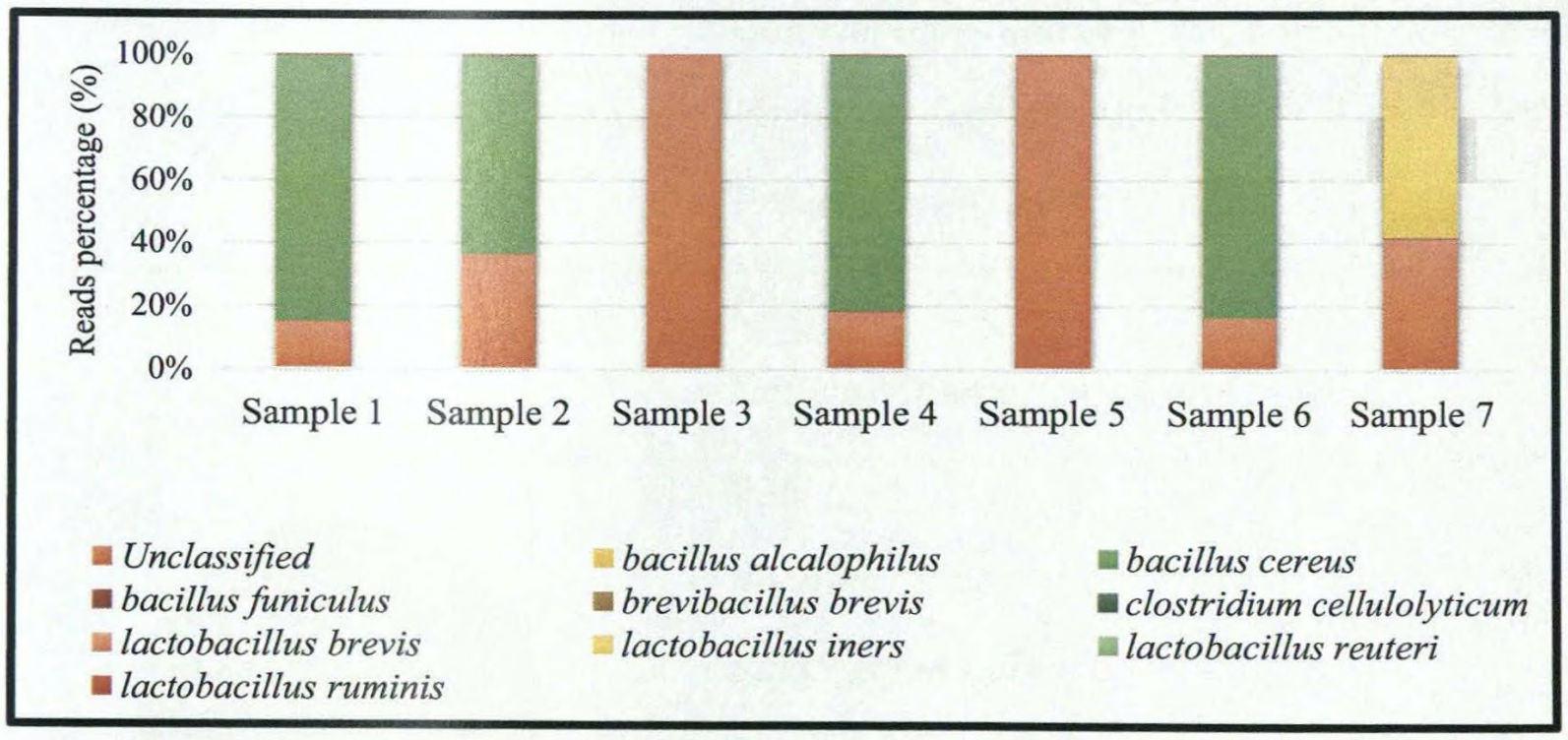
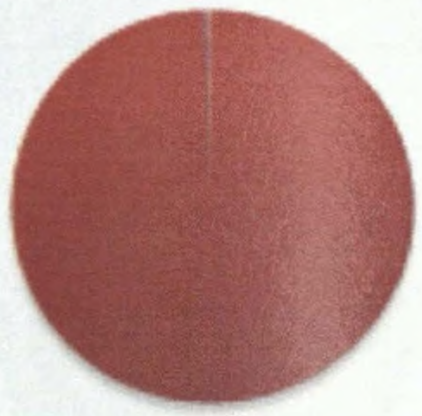


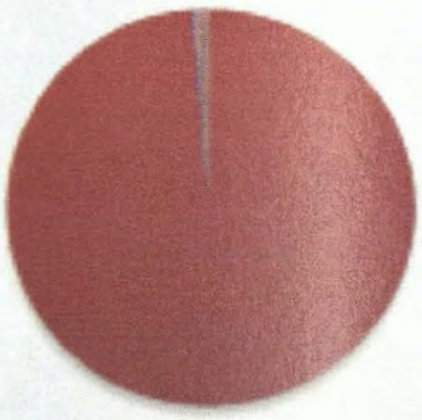
Figure 3.24 Species level classification

Species Level Identification- using Mothur software



P9

Bacillus cereus	99.68%
Bacillus funiculus	0.17%
S. paucimobilis	0.08%
B. alcalophilus	0.07%



P23

Bacillus cereus	98.82%
Bacillus funiculus	0.34%
D. aeronauticum	0.18%
B. alcolophilus	0.16%
Brevibacillus brevis	0.12%
Bacillus coagulans	0.09%
Clostridium cellulolyticum	0.08%
Lactobacillus kimchi	0.06%
Lactobacillus ruminis	0.05%
Caloramator indicus	0.04%
Varivorax paradoxus	0.04%
Prevotella Shahii	0.03%

P26

Not classified up to species level

P30

Bacillus cereus	99.79%
Bacillus funiculus	0.09%
Clostridium cellulyticum	0.08%
Prevotella shahii	0.04%

	P34	
	Not classified up to species level	
	P36	
	*Bacillus cereus*	99.79%
	Bacillus funiculus	0.09%
	Clostridium cellulolyticum	0.08%
	Lactobacillus ruminis	0.04%
	Prevotella shahii	0.03%
	P54	
	*Lactobacillus iners*	97.86%
	Lactobacillus reuteri	0.72%
	Lactobacillus brevis	0.43%
	Lactobacillus acidipiscic	0.40%
	Lactobacillus kimchi	0.13%
	Clostridium cellulotyicum	0.11%
	Collinsella stercoris	0.10%
	Clostridum formioaceticum	0.07%
	Bacillus alcolophilus	0.06%
	Bacillus cereus	0.04%
	Caloramator indicus	0.04%
	Listeria monocytogenes	0.04%

Figure 3.25 Quantitative data obtained at species level

The total number of reads and percentage of each bacteria present in the samples up to species level. Next generation sequencing makes available for quantitative data (read count and percentages) of each species present in the sample.

3.3.1.3 Demographic characteristics of women with pre-eclampsia positive for bacteria

Table 3.16 Demographic characteristics of the seven women with pre-eclampsia

	P09	**P23**	**P26**	**P30**	**P34**	**P36**	**P54**
Age	28	25	19	34	27	26	32
BMI	21	24	19	20	19	23	27
BP(mmHg) at ANC	100/60	120/70	110/70	100/70	120/80	100/80	90/60
Albumin at ANC	nil	nil	nil	nil	nil	nil	nil
Ethnicity	Sinhala	Tamil	Sinhala	Tamil	Sinhala	Sinhala	Sinhala
Mothers Occupation	House Wife	House Wife	House Wife	Pharmacy Assistant	House Wife	Teaching	House Wife
Known Illness/STD/UTI	No	No	Mild Cough	No	Mild Cough	No	No
BP(mmHg) at delivery	140/90	150/100	140/100	140/90	160/100	150/100	150/100
Albumin at delivery	3+	2+	3+	3+	2+	3+	1+
Rise in BP from ANC to delivery	40/30	30/30	30/30	40/20	40/20	50/20	60/40
Late Vs Early Onset PE	Late 36.0	Late 35.0	Early 30.1	Late 38.0	Early 29.4	Late 35.2	Early 31.5
Gestation at delivery	36.0	35.0	30.0	38.3	29.4	35.3	36.5
Indications for LSCS	PE	PE	PE IUGR Low Plt	PE	Severe PE	PE	PE IUGR
Weight of baby at birth (kg)	2.600	1.945	0.480	2.150	1.000	2.318	1.670
Neonatal Death	-	-	**Neonatal Death**	-	**Neonatal Death**	-	-

Demographic characters of the seven with preeclampsia illustrated. BMI; Body Mass Index, BP; Blood pressure, ANC; Antenatal Clinic, STD; Sexually Transmitted Diseases, UTI; Urinary Tract Infection, PE; Preeclampsia, LSCS; Lowe Segment Caesarean section.

As shown in Table 3.16, the 7 women with pre-eclampsia who detected positive for the presence of bacteria were between 19-32 years of age with the BMI between the ranges of 19-27. None of these women had ever smoked and had no known infectious diseases or reported any serious illness during the pregnancy other than being pre-eclamptic. All women were reported to have had normal blood pressure at the antenatal booking and had no signs of albumin. However, all 7 were diagnosed of pre-eclampsia with systolic blood pressure more than 140mmHg and diastolic blood pressure more than 90 mmHg and containing albumin more than 300mg per day. Patient with the screening number P54 had a 60/40mmHg rise in her blood pressure and the rest had more than 30/30mmHg rise in their blood pressure from ANC booking date to the date of delivery.

Except for the patient with the screening number P30, all six of them delivered pre-term and four of them (P36, P34, P26 & P9) had emergency caesarean sections. However, patients P26 & P34 had pre term delivery before 30 weeks and had serious pregnancy complications due to pre-eclampsia. Foetus of these two women suffered from intra uterine growth restriction (IUGR) and low fetal movement (FM) and had to be delivered immediately. The baby born to the woman with screening number P26 weighed 480g at birth and baby of P34 patient was just 1kg. Both these babies died within a few days' of birth due to pre-maturity. Patients P23, P26, P34 & P54 all had babies with birth weight less than 2kg. Table 3.17 below illustrates the bacteria identified in these 7 women in both phase 1 and phase 2 of the study.

Table 3.17 Bacteria identified in the placental tissues of 7 women with pre-eclampsia

#	Phase 1	Phase 2
P9	*Bacillus cereus*	*Bacillus cereus* *Anaerobacillus* *Staphylococcus* *Listeria*
P23	*Bacillus sp.*	*Bacillus cereus* *Anaerobacillus* *Staphylococcus* *Anoxybacillus* *Variovorax paradoxus* *Prevotella shahii*
P26	*Klebsiella pneumoniae* *Proteobacterium*	*Nocardiopsis* *Escherichia* *Shigella* *Salmonella* *Yersinia* *Klebsiella* *Variovorax paradoxus*
P30	*Bacillus sp.* *Bacillus circulans* *Anoxybacillus flavithermus*	*Bacillus cereus* *Anaerobacillus* *Listeria* *Prevotella shahii*
P34	Uncultured bacteria	*Anoxybacillus* *Geobacillus*
P36	*Bacillus sp.* *Anoxybacillus flavithermus*	*Bacillus cereus* *Anaerobacillus* *Listeria* *Prevotella shahii*
P54	Uncultured bacteria	*Dialister* *Anoxybacillus* *Listeria* *Laribacter* *P. endodontalis*

3.3.2 Bacterial identification using 16S Independent Metagenomics NGS

Bacterial detection and identification using the 16S independent metagenomics was carried out by purifying the bacterial DNA by removing human host DNA contaminant using the MolYsis Bacterial Basic kit of Molzem, Germany. However, it was not possible to get substantial amounts of bacterial DNA through this method. Most of the bacteria present in the samples were lost during the process of removing human DNA. It is necessary to remove human DNA contamination to carry out 16S independent metagenomics, which uses the whole genome amplification. Due to this status the 16S independent metagenomics test is not suitable for the detection of bacteria from a human sample as the presence of a large background of human DNA would hinder the identification of bacteria. As explained our attempts for the use of 16S independent metagenomics method to identify bacteria from human sample was not successful.

3.3.3 Interpretation of Results

The results obtained provide preliminary evidence for the presence of infectious agents causing gastroenteritis, respiratory infections and periodontal diseases in association with pre-eclampsia. The results of phase 1 & 2 of the study indicated a significant association of placental bacteria with pre-eclampsia.

4 DISCUSSION

4.1 Phenotypic analysis of the study population

4.1.1 Study population

The study was carried out on 55 primiparous women with pre-eclampsia (case group) and 55 primiparous normotensive pregnant women (control group). The women with pre-eclampsia were matched for age and body mass index (BMI) with the control group. As indicated in table 3.3, mean age of women with pre-eclampsia was 27.5 and the mean age of the normotensive pregnant women was 28. Whilst the mean BMI in women with pre-eclampsia was 22.2, the mean BMI in the control group was 22.8. This signifies the cases and controls recruited to the study were properly matched. Strict defined inclusion and exclusion criteria were followed throughout the patient recruitment and all samples were collected at the time of caesarean section, under strict aseptic conditions.

It was ascertained that women of the study population with pre-eclampsia faced severe pregnancy complications in comparison to the normotensive pregnant women. At the first antenatal booking, both case and control group of women remained with normal blood pressure with no signs of proteinuria. However, as the pregnancy advanced the women with pre-eclampsia developed hypertension with systolic blood pressure of $\geq$ 140mmHg, diastolic blood pressure of $\geq$ 90mmHg and having proteinuria of more than 300mg per day. 30 (54.5%) out of the 55 women with pre-eclampsia developed severe systolic blood pressure, 17 (30.9%) developed severe diastolic blood pressure and 28 (50.9%) of them developed severe proteinuria.

Furthermore, 36 (65.4%) of 55 women with pre-eclampsia delivered preterm, had babies with intra uterine growth restricted (IUGR), low fetal movement and gave birth to babies with very low birth weight. All normotensive women in contrast had uncomplicated pregnancies and underwent delivery through caesarean section due to obstetrics conditions such as maternal request, primi-breech, head not engaged and unfavourable cervix.

A study carried out during the period 1994-2003 with 364 cases with pre-eclampsia and 249 control women had reported that pre-eclampsia is associated with high maternal complications, high rate of IUGR and low birth weights (Liu et al., 2008). Another complication related to severe pre-eclampsia is the occurrence of HELLP syndrome. In the current study 2 (3.6%) women with pre-eclampsia developed HELLP syndrome. However, the incidence of HELLP syndrome in the current study is much less compared to an incidence of 6.4% reported in a study carried out in 2006 (Demir et al., 2006).

A study carried out by (Jesmin, 2011), reported that 64% of women with pre-eclampsia delivered pre-term whilst 48% of babies born to these women were of low birth weight. This study reported a prenatal mortality rate of 8%. It is fascinating to note the rate of complications reported in the study carried out by (Jesmin, 2011) has a close similarity to the rate of complications recorded in the current study.

4.1.2 Maternal morbidity and mortality

Pre-eclampsia is associated with severe maternal and fetal morbidity and mortality (Sarsam et al., 2008, Wagner, 2004, Thangaratinam et al., 2008, Gaillard et al., 2011b, Ghulmiyyah and Sibai, 2012, Dissanayake et al., 2007). Severe maternal morbidity was defined as systolic blood pressure of (SBP $\geq$ 160mmHg), diastolic blood pressure of (DBP $\geq$110 mmHg), proteinuria of $\geq$ 3+ on HCT (> 150mg/dl), thrombocytopenia of (< 100x 10^9/l), impaired liver function (aspartate aminotransferase (AST) and alanine aminotransferase (ALT) of $\geq$ 70 U/L), renal failure requiring dialysis, placental abruption and eclampsia (Dissanayake et al., 2007).

All these clinical features are associated with maternal morbidity and mortality. More than half of the women in the case group were diagnosed with severe pre-eclampsia and developed most of the above morbidities. Research carried out over the years have given clear evidence that early onset pre-eclampsia is more severe than late onset pre-eclampsia (Steegers et al., 2010, Dissanayake et al., 2007). It is evident that the babies born to women with early onset pre-eclampsia carries a higher risk of being born premature and to possess low birth weight. 17 (94%) of women with early onset pre-eclampsia gave birth to babies with a birth weight below 2kg. 14 (77.7%) had babies with birth weights between 1 to 1.5kg and 3 (16.6%) had babies with a birth weight less than 1 kg. Accordingly it is evident that babies born to women with early onset pre-eclampsia had many prenatal complications in comparison to late onset pre-eclampsia ($P < 0.0001$). The overall risk factor for women with pre-eclampsia to give birth to babies with a birth weight less than 2kg is almost 50%.

4.1.3 Rise in blood pressure

The rise in blood pressure is defined as a rise in systolic pressure of >30mmHg or rise in diastolic pressure of >15mmHg above the blood pressure recorded at the first antenatal booking (James and Nelson-Piercy, 2004). Literature suggests that the rise in blood pressure from a pre-pregnancy or an early pregnancy value (SBP of ≥ 30mmHg and /or DBP of ≥ 15 mmHg) would be more accurate than just considering a threshold value as ≥ 140/90 mmHg in defining pregnancy induced hypertension (Australasian-society, 1993, National-high-blood-pressure-educational-programme, 1990, Dissanayake et al., 2007, Redman and Jefferies, 1988, Gaillard et al., 2011a).

Applying the standard definition proposed by the ISSHP or the use of rise in blood pressure in diagnosing pre-eclampsia is presently under much debate with many controversial views being expressed. Whilst some restrict themselves to the standard definition proposed by the ISSHP, certain arguments prevail that a rapid rise in systolic blood pressure of more than 30 mmHg or diastolic blood pressure of more than 15 mmHg should be taken into account in diagnosing preeclampsia (Mustafa et al., 2012).

Duley et al recommends the use of the standard definition in the diagnosis of pre-eclampsia (as mentioned in section 1.1) (Duley et al., 2006). National Institute for Health Services and Clinical Excellence also follows the standard definition put forward by the ISSHP in diagnosing pre-eclampsia (NICE, 2011). However, the British Hypertension Society recommends the use of either the rising blood pressure of ≥ 15mmHg diastolic or ≥ 30mmHg systolic from early pregnancy or diastolic blood pressure ≥ 90mmHg on 2 occasions 4h apart or a diastolic blood pressure of ≥110mmHg on one occasion with 1+ proteinuria as an indication for referral and as the criterion for diagnosis of pre-eclampsia (Williams et al., 2004a, Williams et al., 2004b). After observing the

rise in blood pressure as recorded in the current study, a suggestion is made to use both the rising blood pressure as well as the standard definition of ISSHP in diagnosing pre-eclampsia.

Rising blood pressure is a significant factor associated with severity of the disease and needs to be taken into careful consideration to ensure a proper management of the disease. The very first pre-eclampsia patient recruited to the current study was a young woman of 27 years old with a BMI of 18.0 recorded at the antenatal booking date. She had systolic and diastolic blood pressure of 90/60mmHg on her first antenatal visit and had no signs of proteinuria. However, at 31 weeks of gestation she was diagnosed of pre-eclampsia with a blood pressure of 140/90mmHg and within 4 days, her blood pressure rose to 170/110mmHg with 3+ albumin. She had SGOT level of 47u/l, SGPT of 24 u/l, platelets of 130,000/mm^3 and she developed fits.

Emergency caesarean section was performed and the baby was delivered at 31.4 weeks of gestation with a birth weight of 980g. This patient developed eclampsia and was admitted to the ICU and her baby was admitted to NICU due to prematurity. However, in this instance, there was a rise in blood pressure of 80/50mmHg by the time she delivered the baby. This resulted in life threatening condition for both the mother and the baby. When she was diagnosed with mild pre-eclampsia having a blood pressure of 140/90mmHg, the rise in blood pressure was 50/30mmHg. If an early intervention was made, it would have been possible to prevent the mother developing in to fits and would have prevented her from developing eclampsia.

Under these circumstances, it is necessary to consider a threshold value depending on the rising blood pressure rather than considering a cut off value as 140/90mmHg. It is reported that

systolic and diastolic blood pressure changes from second to third trimester is positively associated with the risks of pregnancy induced hypertension and preeclampsia (Gaillard et al., 2011a). The National High Blood Pressure Education Programme consensus report (NHBPEP, 2000), stresses the position that patients with blood pressure below 140/90 mmHg but have experienced a rise in systolic blood pressure of 30mmHg or rise in diastolic blood pressure of 15mmHg should be managed as high-risk patients for pre-eclampsia (Lindheimer et al., 2010). Leaving an expectant mother to reach the threshold value of 140mmHg may risk the lives of two due to severe maternal and neonatal complications. Early diagnosis and treatment no doubt will reduce the maternal and prenatal morbidity and mortality associated with pre-eclampsia.

4.1.4 Comparing two cohort studies

This is the first time, two similar cohorts (women with pre-eclampsia as case group and normotensive pregnant women as control group) were recruited maintaining same inclusion and exclusion criteria from similar hospitals were analysed and compared in a Sri Lankan population.

Table 3.9 compares the phenotypic data of women with pre-eclampsia recruited between the periods of 2001-2003 and 2010-2011. The mean age of women with pre-eclampsia recruited to the study carried out by Dissanayake et al was 27.1 and the current study was 27.5. The mean BMI of the pre-eclamptic women recruited to the study carried out by Dissanayake et al was 21.0 and was 22.0 in the current study. The mean systolic blood pressure of the women with pre-eclampsia in the 2001-2003 study was 111.7 in comparison to 110.0 in the current study. Diastolic blood pressure was 71.7 in the study carried out in 2001-2003 and was recorded as 73.0 in the present study carried out in 2011-2013. This signifies that both studies even though a decade apart, were performed within a similar study population.

The median gestation age at delivery for the women with pre-eclampsia was 35 (32-38) and 36 (33-37) in the two studies respectively. As illustrated in table 3.9, during the periods of 2010 to 2011 it was observed that 30 of 55 (54.5%) women had systolic blood pressure of ≥ 160 mmHg while in 2001 to 2003 it was 136 of 180 (75.5%). 17 of 55 (30.9%) women suffered from severe diastolic blood pressure of ≥ 110 mmHg during 2010-2011, whilst 151 of 180 (83.3%) during the period of 2001-2003. Proteinuria ≥ 3+ HCT was recorded in 28 (50.9%) women during the period of 2010-2011 and 156 of 180 (86.6%) during 2001- 2003. Accordingly a gradual reduction was observed (between 30-50%) in the pregnancy complications associated with pre-eclampsia over the past 7-10 year period.

There were 106 (58.9%) male babies born during the study carried out in 2001-2003 and 27 (49%) in the study carried out in 2010-2011. In consequent this indicates the baby's gender has no influence on the occurrence of the disease in women with pre-eclampsia.

There is a considerable improvement in maternal life expectancy with one maternal loss in the study carried in 2001-2003 to no maternal deaths recorded in the current study. In addition, as observed by the low rate of complications than in the earlier study the obstetricians seems to be more inclined to intervene early when managing women with pre-eclampsia. For the rate of maternal and prenatal complications to decrease early intervention and improvement in the medical care may have played a vital role. Awareness imparted to the patients during their antenatal visits and being educated by the health professionals on the importance of early diagnosis and treatment could be the main reason behind the low rate of complications recorded in this study in comparison to the previous study.

As reported by Dissanayake et al, 2007, 16 (8.9%) out of 180 women developed eclampsia in 2001-2003 (Dissanayake et al., 2007). Where as in the current study conducted in 2010-2011 there were 8 (14.5%) out of 55 women who developed eclampsia. According to recent literature even today, 12% of all maternal deaths are caused by eclampsia (Ghulmiyyah and Sibai, 2012). A rise in the number of eclampsia patient could result in a negative impact on the management and treatment of pre-eclampsia. This brings out the need for training and educating the health workers on the proper management and treatment of pre-eclampsia. Furthermore, the maintenance of proper standards in diagnosing of pre-eclampsia and a better understanding of the disease aetiology may play a significant role in managing women with pre-eclampsia.

The rise in the number of women who suffered from severe pregnancy complications due to pre-eclampsia may indicate that delaying intervention until blood pressure reaching the threshold value of 140/90mmHg may result in adverse effects on pregnant women. By analysing the results of both the studies (study during 2001-2003 and 2010-2011) it can be recommended that both the threshold value and the rise in blood pressure levels need to be taken into account when treating patients (Dissanayake et al., 2007, Williams et al., 2004a, Williams et al., 2004b, Gaillard et al., 2011a).

When comparing the two studies, a reduction in the maternal and fetal mortality rates over the years could be observed. Ghulmiyyah and Sibai in 2012 report that the number of maternal deaths have fallen progressively during recent years though pre-eclampsia remains the main cause of maternal deaths. Pre-eclampsia continues to remain a life threatening condition to both the pregnant woman and her unborn baby.

As shown in table 3.9, in the current study there were 13 women with pre-eclampsia (23.6%) being admitted to intensive care unit ($P < 0.001$). 29 babies (52.7%) were admitted to neonatal intensive care unit or prenatal baby units as most of them (n=26, 86.6%) were born premature ($P < 0.001$). In the study carried during 2001 to 2003, 69 out of 180 women (38.3%) with pre-eclampsia had preterm delivery before 34 weeks and similarly 18 out of 55 (32.7%) preterm deliveries before 34 weeks were recorded in the study during 2001-2003.

However, in both the studies mean birth weight of babies born to women with pre-eclampsia appeared to be the same. In the current study the mean birth weight of babies born to early onset pre-eclampsia was 1.27 and late on set pre-eclampsia remained at 2.4, similarly, in the study carried out by Dissanayake et al the mean birth weight of babies born to women with early onset pre-eclampsia was 1.13 and 2.3 for late onset pre-eclampsia. As illustrated in the Table 3.9, 27 (49%) of women with pre-eclampsia in the study carried out in 2010-2011 delivered babies with a birth weight below 2kg and one of them delivered a baby with a birth weight of 480g. This emphasises the position that a significant rate of complications associated with pre-eclampsia continues to prevail.

Even though number of women undergoing preterm delivery has improved over the years, it yet remains a matter of concern which requires serious attention. In contrast to the women with pre eclampsia, all normotensive women in both the studies were delivered at term and the circumstances under which caesarean section was performed remains maternal requests, breech position, transverse lie, head not engaged and unfavourable cervix. All babies born to normotensive pregnant women in both studies had a birth weight $\geq$ 2kg and none of them required intensive care treatment.

As reported in the study conducted during 2001-2003, the neonatal intensive care unit (NICU) at the Castle Street Hospital for Women had 15 functioning cots with incubators and four cots with ventilators (Dissanayake et al., 2007). However after a 7-10 year period and at the time the current study was undertaken only 13 functioning cots with incubators and four cots with ventilators were available at this particular hospital. Limitations of facilities available at the treatment hospitals could adversely affect the prenatal mortality rate. During instances where the mother's condition is deteriorating and an emergency caesarean section is required, availability of cots and required facilities would be a deciding factor and remains a matter between life and death.

In contrast to 45 out of 180 (25%) prenatal losses reported in 2001-2003, only 3 out of 55 (5%) prenatal losses were recorded in the current study (Table 3.9). The reduction in both maternal and fetal death rates may be due to the improvements in the medical sector in Sri Lanka, where medical doctors and nursing staff has undergone comprehensive training and education on early diagnosis of pre-eclampsia. Proper management of pre-eclampsia provides an opportunity to avoid pre-term delivery which caused pre-mature births and prenatal loses. Even though the overall prenatal mortality was recorded to be 5% in the current study, all 3 deaths recorded were from women with early onset pre-eclampsia. In other words pre-eclampsia was responsible for 16.6% (3 of 18) prenatal deaths in women with early onset pre-eclampsia ($P < 0.0001$) (Table 3.7). The 5% prenatal death rate is similar to the PNMR in association with pre-eclampsia in western countries (Zhang, 2003, Baldwin et al., 2001). A gradual decrease in prenatal deaths from 25% in 2001 to 5% in 2011 could be observed (Dissanayake et al., 2007). Notwithstanding the fact that conditions are improving compared to the past, a significant number of fetal mortality and maternal morbidity continues to affect the society we live in.

4.2 Molecular Genetic Techniques

4.2.1 Technique used in phase 1 and phase 2

16S and 28S metagenomic tests are among the latest methods that are becoming increasingly popular for the detection of infectious agents and for the phenotypic characterisation of bacteria and fungi (Amann et al., 2000, Eickbush and Eickbush, 2007). Amplification of 16S rRNA gene allows the detection of both viable and dead cells. The method is exceptionally sensitive and capable of detecting microorganisms which under normal conditions were culture negative due to antibiotic treatment, giving them a distinctive advantage over traditional culture based methods (Kemp et al., 2005, Jalava et al., 2001).

16S rRNA gene is 1542bp long (Janda and Abbott, 2007). However sequencing a 500-700bp fragment is sufficient for bacterial identification. The 16S rRNA gene consists of nine variable regions. A significant difference between the techniques used in phase 1 and phase 2, is that in phase 1 only partial 16S rRNA gene was amplified, whereas in phase 2 whole 16S rRNA gene was amplified. BSF 8/20 and BSR 534/18 primers (Cai et al., 2003) were used in phase 1 and a 500bp fragment of the V1, V2, V3 regions of the 16S rRNA gene was amplified for the identification of bacteria to species level. In phase 2, the whole genome of 16S rRNA gene was amplified via the 16S BSF 8/20 and 16S BSR 1541/20 primers which amplified all 9 variable regions (Whiteley et al., 2012).

The medical field took a new direction with the discovery of the structure of DNA by Watson and Crick in 1953 (Watson and Crick, 1953). Subsequently, in 1977 Fredrick Sanger introduced the DNA sequencing method using chain terminating inhibitors, which no doubt

revolutionized the field of genetics (Sanger et al., 1977). What got underway in 1977 as 'Genomics' has today extended to 'Metagenomics', simplifying the identification of microbes in their natural environment (Whiteley et al., 2012, Thomas et al., 2012, Simon and Daniel, 2011, Illumina, 2012b).

In phase 1 of the present study, 16S metagenomics based on Sanger sequencing was used to identify the bacteria present in the placental tissue samples. After amplification, the partial 16S rRNA gene was sequenced by Sanger sequencing also known as capillary sequencing. 500bp portion of the 16S rRNA gene was cloned and sequenced for the identification of bacteria from a mixed population. In 2010, at the time the subject study commenced, 16S metagenomics based on Sanger sequencing was an area not previously explored in Sri Lanka.

Metagenomic sequencing, until recent times was carried out using Sanger sequencing (Petrosino et al., 2009). For a medically meaningful microbial identification, it is imperative to identify bacteria up to genus or species level. However in certain instances, Sanger based metagenomics fails to identify the less frequent organisms from a mixed population due to the low coverage of capillary/ Sanger sequencing (Petrosino et al., 2009).

Next generation sequencing, a technique introduced just a few years back has overcome limitations experienced in the past and has displayed a capability to generate a complete classification of the total microbiome present in a particular sample (Mende et al., 2012, Kunin et al., 2008, Petrosino et al., 2009). Accordingly, under phase 2 of the subject study 16S

metagenomics was employed via next generation sequencing technology. The process facilitated the identification of all bacteria that were present in the sample.

Two acknowledged approaches are available for the metagenomic next generation sequencing, adapted for the identification of infectious agents. The first approach is the use of 16S metagenomics where the 16S rRNA gene is amplified and sequenced. The second approach is the 16S independent metagenomics, where the whole genome of bacteria is amplified and sequenced (Petrosino et al., 2009, Whiteley et al., 2012). The second approach is the most suitable for environmental samples whilst the first approach remains the best for biological samples rich in human DNA with lower number of bacterial cells.

4.3 Association of Infectious Agents with Pre-eclampsia

4.3.1 Infectious agents identified in phase 1

The subject study provides evidence of the presence of bacteria in the placental tissues of women with pre-eclampsia signifying a logical relevance to the association of infectious agents in the multifactorial aetiology of pre-eclampsia. All subjects recruited for the study were primiparous and underwent elective caesarean section. All samples were collected soon after delivery following stringent aseptic conditions. Such steps were taken to avoid every possible microbial contamination and for the diminution of any errors. None of the women with pre-eclampsia or the controls recruited for the study experienced labour or underwent labour induction. Such circumstances eliminated the risk of bacterial contamination during labour.

As mentioned in section 3.2.7, at the end of phase 1 of the subject research project, from the placental tissue samples obtained from 55 women with pre-eclampsia, 7(12.7%) women were positive for the presence of bacteria (P=0.006). 1(1.8%) was detected positive for the presence of fungi and none (0%) were detected positive for the presence of viruses. None (0%) of the normotensive women samples were detected positive for the presence of bacteria, fungi or viruses.

It is exciting to deliberate on the role of the infectious agents identified in the study on the development of pre-eclampsia. The fungus strain identified was *Malassezia restricta.* It is a commensal of normal skin flora and dandruff (Ashbee, 2007) and is a possible contaminant in-spite of taking stringent aseptic conditions and this down plays its significance. Therefore at

the end of phase 1, it was concluded that fungi and viruses are not associated with the aetiology of pre-eclampsia.

Based on information gathered in phase 1 of the study it is discerned, *Bacillus* sp. (57.1%) was the most abundant (present in sample P9, P23, P30 & P36) and *Bacillus cereus* was present in 1 sample (14.2%) P9. It is a gram positive, spore forming rod shaped bacteria associated with tissue destruction and reactive exoenzyme production. The protein enterotoxin produced by *Bacillus cereus* causes diarrhoea and food poisoning (Bottone, 2010, Didelot et al., 2009). *Bacillus circulans* was present in 1 sample (14.2%) P30 and similar to *Bacillus cereus* is known to cause diarrhoea and has a history of urinary tract infection (Alebouyeh et al., 2011). *Bacillus circulans* also displays certain similarities to *Bacillus cereus* and is also a gram positive, spore forming rod belonging to the phylum fermicutes.

Klebsiella pneumonia belongs to the phylum proteobacterium and is a gram negative bacterium, which is also reported to cause pneumonia. It was also reported that *K. pneumonia* is responsible for early pregnancy loss and neonatal deaths. *K. pneumonia* was detected in sample P26 and is believed to be responsible for premature rupture of membranes (Torabi et al., 2008, Omwandho et al., 2006).

Stenotrophomonas was also identified in sample no P23. It is a gram negative bacterium belonging to the phylum proteobacterium and is responsible for pneumonia and urinary tract infection. *Anoxybacillus flavithermus* was detected in sample P30. Although generally considered non-pathogenic, lately, it has been discovered to be a contaminant of dairy products

and to be responsible for food spoilage (Burgess et al., 2010). The bacteria identified in 6 samples (85.7%) belonged to the phylum fermicutes and only 3 samples (42.8%) had bacteria belonging to phylum proteobacterium. 4 women out of the 7 when inquired stated that they experienced a mild cough during the pregnancy. However, surprisingly none of these women detected positive neither complained of having any gastro-intestinal symptoms for infection.

4.3.2 Infectious agents identified in other biological samples

At the end of phase 1, there were positive indications of the presence of bacteria and fungi in the placental tissue samples collected from women with pre-eclampsia. Accordingly the study was further extended to cover the other biological samples (blood, urine and amniotic fluid) collected at the same time placental tissues were obtained for the study. Only sample P9 was positive for the presence of bacteria in the amniotic fluid samples and was found to be *Bacllus cereus.* A noteworthy fact of this result was the observation that the placental tissue sample obtained from the same patient carried *Bacllus cereus*. This signifies the possibility of bacteria (*Bacllus cereus*) which was present in the placental tissue sample entering the amniotic cavity.

Even though 7 placental tissue samples were positive for the presence of bacteria, only in one patient the bacteria was found to have invaded the amniotic fluid. Similarly, a study carried out in the USA for the detection of microbial invasion of the amniotic cavity (MIAC) in women with preeclampsia found that the prevalence of MIAC in preeclampsia is low and suggests that intra-amniotic infection plays only a limited role in preeclampsia (DiGiulio et al., 2010). Although there were preliminery evidence for the placental bacteria to be associated with pre-eclampsia (P=0.006), the presence of bacteria in the amniotic fluid were not significant in women with pre-eclampsia (P= 0.332) (Table 3.13).

3 blood samples (P7, P8, P10) were positive for the presence of bacteria (Table 3.11). *Reyranella massiliensis* was detected both in samples P7 & P8. *Reyranela* was recently found to be a bacterium isolated in freshwater and is thought to be non pathogenic (Pagnier et al., 2012). *Ralstonia picketti* detected in sample P10 is a part of the commensal flora of the oral cavity and upper respiratory tract. Infection in healthy individuals is extremely rare but could act as an infectious agent in immunocompromised patients (Stelzmueller et al., 2006). Further, Dungan Roberts in 2011, reports that *Ralstonia picketti* causes life threatening infections in immunocompromised patients (Dungan, 2012). The baby born to the woman detected with *Ralstonia picketti* died after a few days of birth due to prematurity and failure of lungs indicates a possible association infection with *Ralstonia picketti.*

Bacteria were also detected in urine samples of 8 (14.5%) women with pre-eclampsia and 9 (16%) normotensive pregnant women. *Lactobacillus gasseri, Lactobacilus crispatus* and *Veillonella montpellieresis* was found in the urine samples of women with pre-eclampsia and *Prevotella bivia, Atopobium vaginae* and *Streptococcus agalactiae* were discovered in urine samples of normotensive pregnant women. All these bacteria discovered in urine were normal flora present in female vagina and are non pathogenic. The presence of bacteria in the urine samples of women with pre-eclampsia were also statistically non significant (P=0.960). The phase 1 of the study provided preliminary evidence of the presence of placental bacteria in association with pre-eclampsia and the association was statistically significant (P= 0.006).

4.3.3 Infectious agents identified in phase 2

As mentioned in the previous section, at the end of phase 1, there was preliminary evidence of a significant association of placental bacteria with pre-eclampsia. However, using Sanger sequencing, it was not possible to get a complete classification of the total microbiome present in the sample. Also, in sample P34 bacteria could not be identified even up to genus level. Accordingly in phase 2 of the study the whole 16S ribosomal RNA was amplified and was sequenced on an Illumina MiSeq next generation sequencing platform. As shown in section 3.3.2, the results obtained in phase 2 were very similar to phase 1 but more specific. Similar to phase 1, *Bacillus cereus* was detected in samples P9, P23, P30 & P36 in phase 2 as well. As already stated *Bacillus cereus* was found to be associated with diarrhoea, food poisoning, pneumonia and lung infections (Didelot et al., 2009, Bottone, 2010). Samples P9, P23 was identified with trace amounts of *Staphylococcus* and samples P9, P30, P54 had *Listeria*. Both *Listeria* and *Staphylococcus* are believed to cause food borne infections (Argudin et al., 2010, Hennekinne et al., 2010, Arakawa et al., 2008).

Sample P26 was detected with *Escherichia, Shigella, Salmonella* which are bacteria known to cause food poisoning (Edwards, 1999, Saito et al., 2005). In addition, *Nocardiopsis* and trace amounts of *Klebsiella pneumonia* which cause respiratory infections (Ko et al., 2002) were also detected in sample P26. However, the baby born to this patient (P26) died as a result of prematurity and non-development of lungs. In addition evidence does exist to suggest an association of *Staphylococcus* and *Klebsiella* species in periodontitis (Fritschi et al., 2008, Kumar et al., 2005, Baehni and Guggenheim, 1996, Consuegra et al., 2011). Poor oral hygiene and periodontal disease may promote oropharyngeal colonization of potential respiratory bacteria such as *Klebsiella pneumoniae* (Scannapieco and Mylotte, 1996).

The samples P9, P23 and P30 was detected with *Anaerobacillus* a bacteria believed to cause periodontal diseases (Loesche, 1991, Booth et al., 2004, Lovegrove, 2004) and in sample P54, Dialister was discovered, a bacteria recently discovered and known to cause periodontitis (Contreras et al., 2000, Doan et al., 2000). Literature points out that poor oral sanitation results in periodontitis and allows the colonization of respiratory bacteria increasing the risk of developing pneumonia and associated lung infections (Scannapieco and Mylotte, 1996).

Although infectious agents responsible for gastroenteritis, respiratory and periodontal infections were discovered from the group of women with pre-eclampsia, it is compelling to note that none of these women complained of diarrhoea or any other major ailment. However, 4 out of the 7 women complained of a mild cough during pregnancy. A possible cause could be that in spite of the fact that bacteria like *Bacillus cereus, Salmonella, Klebsiella* were present, to develop symptoms a significant number of bacterial cells are involved. Many of these infections caused by foodborne bacteria may be asymptomatic. More often acute gastroenteritis is mild and may go unnoticed.

Gastroenteritis in humans may vary depending on the serotype, the strain and the dose of bacterial cells. For an example, an oral dose of at least 10^5 *Salmonella typhi* cells are required to cause typhoid and 10^9 *Salmonella* cells to cause symptoms of toxic infections (Todar, 2008). Accordingly, it could be argued that most of the *Salmonella* infections are asymptomatic and may go unnoticed (Jertborn et al., 1990). This may explain the reason seven women detected positive for the presence of infectious agents never complained of an infection during their pregnancy.

Staphylococcal enterotoxemia caused by *Staphylococcus aureus* is reported to be related to pre-eclampsia (BGI, 2014). A study carried out in 2010 in Canada reports that *Salmonella* has the ability to localize into deeper layers of the placental tissue. The nature of inflammation triggered by the infectious agents strongly affects the abnormal placentation and host survival (Chattopadhyay et al., 2010). Chattopadhyay further states that *Salmonella* species causes trans-placental fetal infections which may lead to pregnancy complications such as preterm labour and preeclampsia.

In a study carried out in India an increased incidences of pre-eclamptic toxaemia (PET) [RR 3.79, 95% CI 1.80-7.97] was detected in women with asymptomatic bacteriuria. *Escherichia coli, Staphylococcus aureus and Klebsiella sp.* were among the organisms identified and the study concluded if urinary tract infection was detected in late pregnancy it might lead to various maternal and neonatal complications like pre-eclampsia, pre-term labour, premature rupture of membrane, intra uterine growth restriction and low birth weight (Jain et al., 2013).

The current study discovered traces of periodontal bacteria in samples with screening no's P23, P26, P30, P36, and P54. As indicated *Anaerobacillus, Variovorax paradoxus, Prevotella shahii, Porphyromonas endodontalis* and *Dialister* were among the periodontal bacteria discovered from the placental tissue samples in the 7 women with pre-eclampsia.

Many recent studies suggest a positive association of periodontitis and pregnancy complications. Contreras reported that chronic periodontal disease and the presence of *Porphyromonas gingivalis, Tannerella forsythensis,* and *Eikenella corrodens* had a significant

association in women with pre-eclampsia (Contreras, 2006). *Porphyromonas* species was also identified in sample no 54 of the current study and all 7 samples of women with pre-eclampsia were positive for periodontal bacteria. The discovery demonstrates that in spite of the fact that the presence was in trace amounts, periodontal bacteria may play a role in the disease aetiology of pre-eclampsia (Jamieson et al., 2009, Sakamoto et al., 2004, Lombardo Bedran et al., 2012, Ghayoumi et al., 2002). Contreras argues that oral bacteria may enter the maternal and fetal blood circulation that would trigger placental inflammation which eventually causes abnormal placentation and the clinical manifestations of preeclampsia (Contreras, 2006). It is further emphasised that periodontal disease in pre-eclampsia exerts a vascular stress to the mother, placenta, and foetus. Periodontal disease may increase the endotoxin, inflammatory cytokines and oxidative stressors at maternal and fetal interface in pregnant women (Contreras, 2006). Such conditions would unequivocally lead to pregnancy complications such as pre-eclampsia. Some of the recent case control studies point out the association of periodontal bacteria in the aetiology of pre-eclampsia. Two studies independently carried out in Korea and Netherlands have discovered periodontal bacteria to be associated with severe pre-eclampsia (Ha et al., 2011, Kunnen et al., 2007). These studies indicate a possible link of periodontal bacteria with pre-eclampsia.

Two hypotheses have been described in explaining the disease mechanism pertaining to periodontitis and pre-eclampsia. The first hypothesis suggests that periodontal disease affects the maternal and fetal immune responses systemically. The second that oral bacteria may enter maternal blood circulation and could directly enter the placental bed, causing localized inflammation and adverse pregnancy outcome in the presence or absence of clinical periodontitis. The study further emphasises that even the commensal species of oral cavity could be associated with pre-eclampsia upon oral-uterine transmission (Han, 2011).

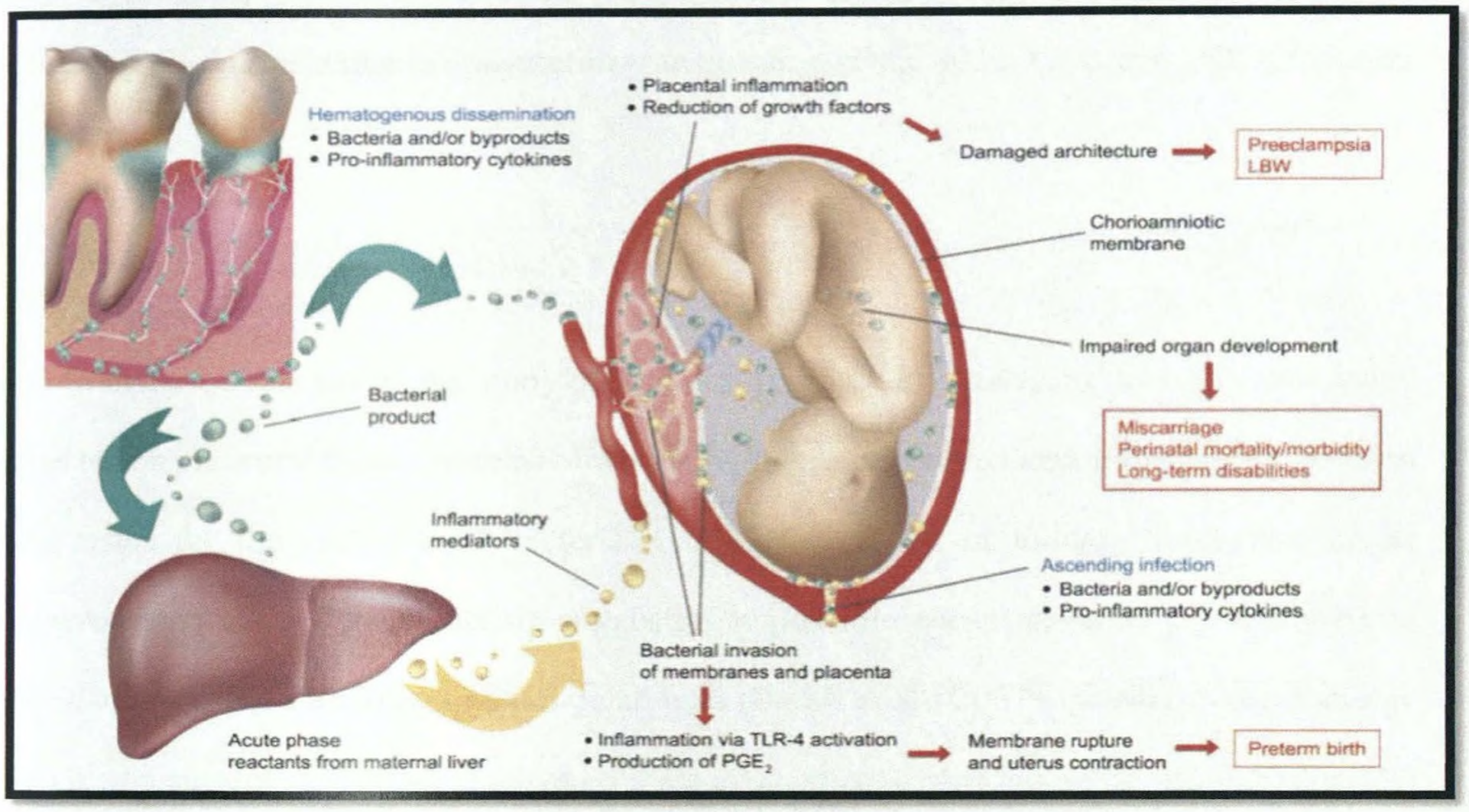


Figure 4.1 Possible pathways associating periodontal disease & pregnancy complications
Foetal exposure to periodontal pathogens may increase inflammatory response in the placenta leading to abnormal placentation causing preeclampsia (Madianos et al., 2013)

Figure 4.1, illustrates a possible biological pathway associating periodontal disease and pregnancy complications. Madianos et al suggests that foetal exposure to periodontal bacteria and/or their by-products (toxins) will result in increased inflammatory response in the placenta, which leads to tissue damage and abnormal placentation. This impairs the nutrition transportation to the placenta and to the foetus. Also the increased inflammatory factors could result in increasing maternal blood pressure causing pre-eclampsia (Madianos et al., 2013).

The literature available suggests that periodontal bacteria play a significant role in the multifactorial aetiology of pre-eclampsia (Madianos et al., 2013, Boggess et al., 2003, Canakei, 2004, Canakci et al., 2004, Canakci et al., 2007, Contreras, 2006, Contreras et al., 2000, Kunnen et al., 2007, Kunnen et al., 2010). Many studies including the study discussed in this

thesis reported that commensal species of oral cavity and periodontitis bacteria could increase the risk of pre-eclampsia due to oral-uterine transmission (Han, 2011, Contreras, 2006, Canakci et al., 2007).

Apart from the current study, the study carried out by Barak et al remains the only other study carried out on placental tissue samples of women with pre-eclampsia and normotensive women which tested for the presence of bacteria. Barak et al found periodontal bacteria such as *Porphyromonas* sp. & *Prevotella* sp, suggesting a possible contribution of periopathogenic bacteria to the disease aetiology of pre-eclampsia (Barak et al., 2007). Similar to the findings of Barak, the current study also discovered trace amounts of *Porphyromonas* sp. & *Prevotella* sp. in women with pre-eclampsia.

Along with the infectious agents causing periodontitis, infectious agents responsible for respiratory and food borne infections were also discovered in the current study. Accordingly and after scrutinizing literature and other study findings, a hypothesis could be arrived at that the presence of bacteria and bacterial endotoxins in the systemic circulation may induce pro-inflammatory cytokine production (Scannapieco, 2004). The activation of inflammatory cells may result in endothelial dysfunction (Faas and Schuiling, 2001, Redman and Sargent, 2005, Roberts and Gammill, 2005, Kunnen et al., 2010). Past research reveals an increased inflammatory responses during pre-eclampsia compared to normal pregnancy (Kunnen et al., 2010). It is believed that alterations in inflammatory responses and endothelial dysfunction results in the underlying aetiology of pre-eclampsia (Kunnen et al., 2010, Sargent et al., 2006).

4.3.4 Antibiotic treatment

Untreated infections pose a great risk to both the mother and her unborn baby. Treatment at an early stage may prevent the adverse pregnancy sequels. Even so, many pregnant women fear taking antibiotics due to possible harmful effect some antibiotics have on the foetus. With the advancement in the field of pharmaceuticals many non-teratogenic antibiotics have been introduced that are safe for the foetus, taken under proper medical guidance and advice (Mylonas, 2011).

The oldest and the safest antibiotic known so far is penicillin. It lyses the bacterial cell wall and cross the placental barrier. At therapeutic doses, penicillin is nontoxic to humans and poses no teratogenic effect (Mylonas, 2011). β-lactam antibiotics such as penicillin & cephalosporines inhibit all strains of *Bacillus*. However *Klebsiella pneumoniae* varies in susceptibility.

Amoxicillin is used against *Anaerobacillus* and *Listeria* whilst for the treatment of *Shigella* ampicillin is used (Bhattacharya and Sur, 2003). Penicillin, amoxicillin and cephalosporin are harmless to the unborn child if the correct dose is administered (Mylonas, 2011). Penicillin, Ampicillin, Amoxicillin are recommended for periodontal bacteria like *porphyromonas* sp. & *Prevotella* sp. In severe cases a combination of amoxicillin and metronidazole is recommended (Kapoor et al., 2012). Further, Amoxicillin is also recommended for the treatment of *Dialister* sp. (Morio et al., 2007).

Accordingly, β-lactam antibiotics such as penicillin & cephalosporin's can be considered the best choice of antibiotics in treating infections in women with pre-eclampsia. Early detection and treatment of an infection may prevent the adverse pregnancy complications faced by women with pre-eclampsia. A randomized trial is required to justify antibiotic treatment as a preventive strategy in clinical management of pregnancy related complications (Todros et al., 2006). It is important to carry out research on this aspect and test antibiotics on animal models to observe the effect of antibiotics on the disease progression of pre-eclampsia.

4.3.5 Improved sanitation/personal hygiene and the reduction of pre-eclampsia

Bacteria causing gastroenteritis, respiratory infections and periodontitis were among the bacteria discovered from placental tissue samples. In the current study this supports the role of bacteria in the multifactorial aetiology of preeclampsia to a greater degree. Accordingly poor oral sanitation increases the occurrence of periodontitis, a local chronic bacterial infection in the oral cavity. This may contribute to endothelial dysfunction increasing the risk of preeclampsia (Cousens et al., 2011). Adding to the disease burden Scannapieco and Mylotte also reports that poor oral hygiene allows the colonization of respiratory pathogens (Scannapieco and Mylotte, 1996). This brings out the need for educating the pregnant women on good personal hygiene practices and the importance of maintaining cleanliness at all times. A cross sectional study that was carried out on 1350 subjects revealed that there was a significant reduction in periodontitis with improved socioeconomic levels (Heine et al., 2003).

Literature suggests that preeclampsia is a disease common to developing countries and countries with low socioeconomic level (Osungbade and Ige, 2011). This indicates that factors such as socioeconomic status, psychosocial factor and life style may also have an effect on the development of preeclampsia. Socio economic condition and female education are few essential factors governing women's health (de Groot et al., 2005).

A study carried out by Silva et al using 3547 pregnant women found that women with low educational levels were more likely to develop preeclampsia (odds ratio 5.12; 95% CI 2.20,11.93) than women with good educational level (Silva et al., 2008). This study strongly pointed out that low maternal socioeconomic status is a strong risk factor for preeclampsia (Silva et al., 2008). Similarly, a study carried out by Lindquist et al found that compared to the

women with higher professional socioeconomic level, unemployed women were at a higher risk of severe maternal morbidity (Lindquist et al., 2013). It is reported that socioeconomic status is associated with health problems such as low birth weight and hypertension (Zhang et al., 2003). Low socio economic status is associated with poor personal hygienic levels, poor environmental conditions and is responsible for the burden of morbidity and mortality associated with the rapid spread of infections (Seneviratne and Rajapaksa, 2000, Zhang et al., 2003).

Unsafe water, contaminated food and poor personal sanitation practices of food handlers may critically contribute to the transmission of infectious agents causing gastroenteritis and other serious repercussions (Morris, 2013). Sanitation is a cornerstone of public health (NHBPEP, 2000). Improvements to water quality, clean environments, healthy food and introduction of clean toilets may in turn lead to a rapid decline in transmitting diseases by reduced exposure to infectious agents. Accordingly, simple remedies such as washing hands with a proper sanitizer, maintaining good oral and body health may be able to decrease the incidence of preeclampsia to a greater extend.

Prevention is better than cure; as this famous idiom points out, to prevent and to lower the risk of preeclampsia we recommend that maternal hygiene and personal sanitation practices are improved and maintained at all times.

4.4 Limitations and future directions

One limitation of this study is the time at which the placental tissue samples were collected. Even though pre-eclampsia is a condition which has its origin in defective placentation in early pregnancy, the placental tissue samples were obtained at the time of delivery a few months later due to ethical and technical reasons. The criteria therefore create the possibility of other bacteria being cleared from the placental bed with the time duration. It is a known fact that certain bacteria have a tendency to pass out over a time lapse and what we report may be an underestimation. This could be another reason for detecting only trace amounts of certain bacteria. However, there is no other means of collecting placental tissue samples other than at the time of delivery.

The study provides evidence of the presence of infectious agents/ bacteria in association with pre-eclampsia. Under such circumstances a possibility exists for the non teratogenic antibiotics to reduce the risk of developing pre-eclampsia. Due to the detrimental effect certain antibiotics causes to the foetus it is not possible to test it directly on pregnant women. Consequently it is possible to test the influence of different antibiotics on pre-eclampsia using an animal model. Mouse is an excellent model for such experiments as it shares many similarities to the human genome. Also clinical manifestations associated with pre-eclampsia or any other disease could be artificially developed in a mouse.

5 CONCLUSIONS

Pre-eclampsia is associated with severe maternal and fetal morbidity and mortality. Good antenatal care, early diagnosis, appropriate patient management with timely delivery of the baby may reduce the undesirable conditions associated with pre-eclampsia. With high maternal and fetal morbidity and mortality associated with pre-eclampsia, it is necessary to find markers for early detection and encourage research of new therapies.

The main focus of this research project was to comparatively study the viruses, bacteria and fungi present in the placental tissues of women with pre-eclampsia and normotensive women using molecular genetic techniques. To our knowledge, this is the first study to use the latest next generation sequencing technology to detect and identify infectious agents present in placental tissues of women with pre-eclampsia. The study was implemented in two phases.

Phase 1 employed 16S, 28S and viral metagenomics using Sanger sequencing to detect and identify bacteria, fungi & viruses present in the biological samples of women with pre-eclampsia and normotensive pregnant women. At the end of phase 1, seven (12.7%) women with pre-eclampsia were detected positive for the presence of bacteria. One (1.8%) was positive for fungi and none (0%) were positive for viruses from the placental tissue samples obtained from 55 women with pre-eclampsia. None (0%) of the normotensive women samples were positive for bacteria, fungi or viruses. Detection of bacteria in the placental tissue samples were statistically significant (P=0.006). Accordingly, phase 1 of this study concluded by providing statistically significant association of bacteria in placental tissue of women with pre-eclampsia.

In phase 2 of the study 16S metagenomics was performed under next generation sequencing for the detection and identification of bacteria. Consequently the whole 16S ribosomal RNA gene (1500bp) was amplified and was sequenced on an Illumina MiSeq personal genome sequencer. This study stands as the first of its kind in Sri Lanka to employ 16S metagenomics next generation sequencing for the detection and identification of bacteria. At the end of phase 2, in detail classification of the complete microbiome present in the 7 positive samples of women with pre-eclampsia was obtained along with the percentages of each bacterium that was present. Based on results achieved, bacteria responsible for gastroenteritis, respiratory and periodontal infection were identified.

It is hypothesized that the presence of bacteria and bacterial endotoxins in the systemic circulation may induce pro-inflammatory cytokines and activation of inflammatory cells. The condition would further triggers the up-regulation of sFLT1 (soluble forms like tyrosine kinase 1), directly influencing the reduction of placental growth factor (PIGF) and vascular endothelial growth factor (VEGF) resulting in an anti angiogenic state causing placental hypoxia, which ultimately would lead to endothelial dysfunction and abnormal placentation that will impair the transportation of nutrition and oxygen, causing an increase in maternal blood pressure leading to pre-eclampsia.

In conclusion, this study confirms the presence of bacteria in the placental tissues of a subset of women with pre-eclampsia and support the role of bacteria in the multi factorial aetiology of pre-eclampsia.

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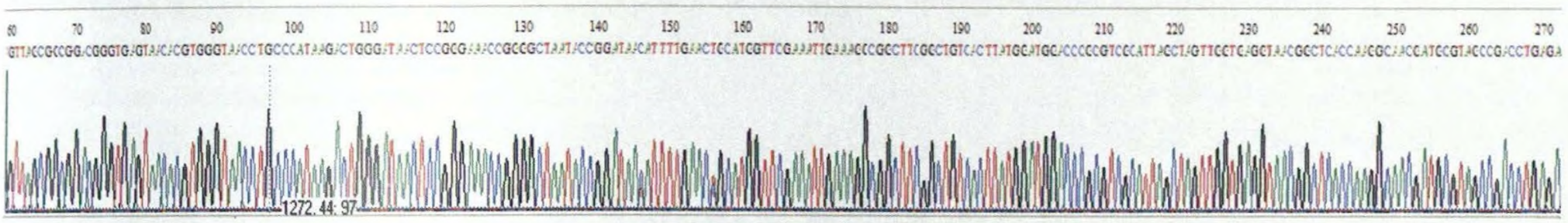
7 ANNEXURES

7.1 BLAST REPORTS

Placental Tissues- P 09



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TAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGC

BLAST report of sample 09

Placental tissue 09

RID M9PFH27W01R (Expires on 04-10 13:26 pm)
Query ID lcl|73931
Description None
Molecule type nucleic acid
Query Length 502

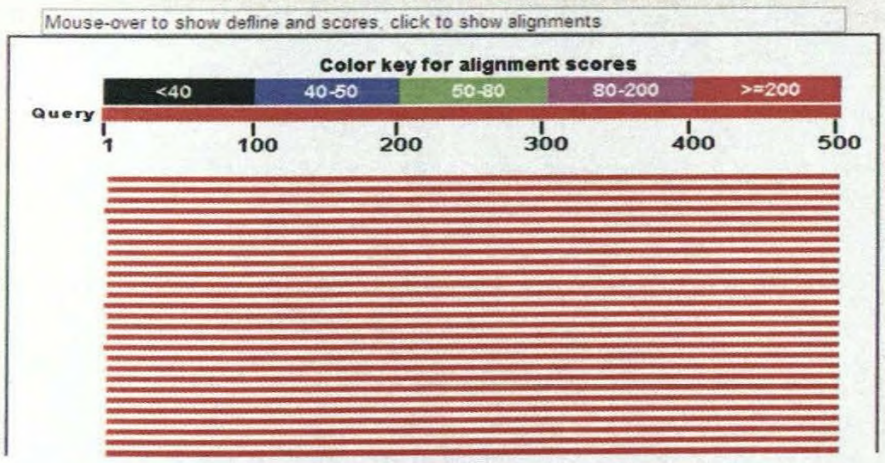
Database Name nr
Description Nucleotide collection (nt)
Program BLASTN 2.2.29+ ▹ Citation

Other reports: ▹ Search Summary [Taxonomy reports] [Distance tree of results]

⊖ Graphic Summary

Distribution of 100 Blast Hits on the Query Sequence

Mouse-over to show defline and scores, click to show alignments



blast.ncbi.nlm.nih.gov/Blast.cgi

Bacillus cereus strain MRBI5 16S ribosomal RNA gene, partial sequence	894	894	99%	0.0	99%	JX028146.1
Bacillus cereus strain AIMST 1MESL 16S ribosomal RNA gene, partial sequence	894	894	99%	0.0	99%	JQ311925.1
Bacillus cereus strain 7R fA 16S ribosomal RNA gene, partial sequence	894	894	99%	0.0	99%	JN626209.1
Bacillus cereus strain RJ1 16S ribosomal RNA gene, partial sequence	894	894	99%	0.0	99%	JN159662.1
Bacillus sp. Q-E1 16S ribosomal RNA gene, partial sequence	894	894	99%	0.0	99%	JN613477.1
Bacillus cereus strain 1142 16S ribosomal RNA gene, partial sequence	894	894	99%	0.0	99%	JF322975.1
Bacillus cereus strain WAS4-2 16S ribosomal RNA gene, partial sequence	894	894	99%	0.0	99%	JF496513.1
Bacillus cereus strain S652Ba-150 16S ribosomal RNA gene, partial sequence	894	894	99%	0.0	99%	HQ238600.1
Bacillus cereus strain S643Ba-136 16S ribosomal RNA gene, partial sequence	894	894	99%	0.0	99%	HQ238562.1
Bacillus cereus strain H5B-366 16S ribosomal RNA gene, partial sequence	894	894	99%	0.0	99%	HQ238529.1
Bacillus cereus strain JY5 16S ribosomal RNA gene, partial sequence	894	894	99%	0.0	99%	HQ833024.1
Bacillus sp. enrichment culture clone SYW13 16S ribosomal RNA gene, partial sequence	894	894	99%	0.0	99%	FJ601643.1
Bacillus sp. enrichment culture clone SYW1 16S ribosomal RNA gene, partial sequence	894	894	99%	0.0	99%	FJ601631.1
Bacillus sp. cp-h50 16S ribosomal RNA gene, partial sequence >gb|JX849099.1| Bacillus sp. H51 16S ribosomal RNA gene, partial sequence	894	894	99%	0.0	99%	EU584547.1
Bacillus sp. cp-h48 16S ribosomal RNA gene, partial sequence	894	894	99%	0.0	99%	EU584546.1
Bacillus sp. cp-h46 16S ribosomal RNA gene, partial sequence	894	894	99%	0.0	99%	EU584545.1

blast.ncbi.nlm.nih.gov/Blast.cgi#393191919

Download ~ GenBank Graphics ▼ Next ▲ Previous Descriptions

Bacillus cereus strain MRBI5 16S ribosomal RNA gene, partial sequence

Sequence ID: gb|JX028146.1| Length: 960 Number of Matches: 1

Related Information

Range 1: 12 to 507 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
894 bits(484)	0.0	494/498(99%)	3/498(0%)	Plus/Plus

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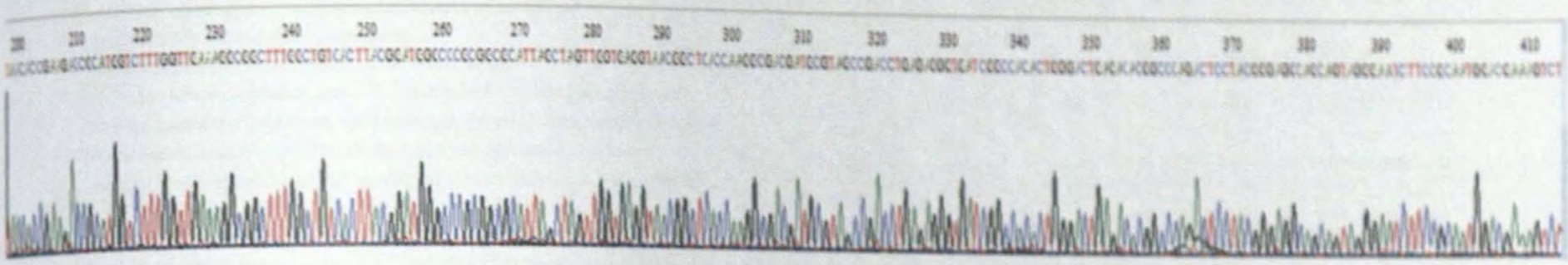
Placental tissue- P 23 (a)



Fasta seq

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GAAAGCCACGGCTAACTACGTG

BLAST report of sample 23a

blast.ncbi.nlm.nih.gov/Blast.cgi

Uncultured bacterium clone nbr257h07c1 16S ribosomal RNA gene, partial sequence	726	726	100%	0.0	97%	HM813014.1
Uncultured bacteria clone F2-31 16S ribosomal RNA gene, partial sequence	726	726	100%	0.0	97%	AY096166.1
Bacillus sp. SSCA19 gene for 16S rRNA, partial sequence, strain: SSCA19	726	726	100%	0.0	97%	AB210933.1
Bacillus sp. SSCA20 gene for 16S rRNA, partial sequence, strain: SSCA20	726	726	100%	0.0	97%	AB210932.1
Bacillus sp. partial 16S rRNA gene, isolate ISR04	726	726	100%	0.0	97%	Y14144.1
Anoxybacillus sp. PS2 16S ribosomal RNA gene, partial sequence	723	723	100%	0.0	97%	KF193531.1
Uncultured Bacillaceae bacterium clone 2247 16S ribosomal RNA gene, partial sequence	721	721	100%	0.0	97%	KF503073.1
Uncultured Bacillaceae bacterium clone 20G-e1 16S ribosomal RNA gene, partial sequence	721	721	100%	0.0	97%	KC918294.1
Uncultured Bacillus sp. clone 12CP-20 16S ribosomal RNA gene, partial sequence	721	721	100%	0.0	97%	KC918004.1
Uncultured Bacillaceae bacterium clone 18G-255 16S ribosomal RNA gene, partial sequence	721	721	100%	0.0	97%	KC917735.1
Uncultured Bacillaceae bacterium clone 16H-22 16S ribosomal RNA gene, partial sequence	721	721	100%	0.0	97%	KC917709.1
Uncultured Bacillaceae bacterium clone 16H-14 16S ribosomal RNA gene, partial sequence	721	721	100%	0.0	97%	KC917703.1
Uncultured Bacillaceae bacterium clone 16H-13 16S ribosomal RNA gene, partial sequence	721	721	100%	0.0	97%	KC917702.1
Uncultured Bacillaceae bacterium clone 16H-10 16S ribosomal RNA gene, partial sequence	721	721	100%	0.0	97%	KC917699.1
Uncultured Bacillaceae bacterium clone 16H-8 16S ribosomal RNA gene, partial sequence	721	721	100%	0.0	97%	KC917697.1
Uncultured Bacillaceae bacterium clone 16H-5 16S ribosomal RNA gene, partial sequence	721	721	100%	0.0	97%	KC917694.1
Uncultured Bacillaceae bacterium clone 16H-2 16S ribosomal RNA gene, partial sequence	721	721	100%	0.0	97%	KC917693.1
Uncultured Bacillaceae bacterium clone 16H-1 16S ribosomal RNA gene, partial sequence	721	721	100%	0.0	97%	KC917692.1

blast.ncbi.nlm.nih.gov/Blast.cgi#37950933

Download ▾ GenBank Graphics ▼ Next ▲ Previous ▲ Descriptions

Bacillus sp. partial 16S rRNA gene, isolate ISR04

Sequence ID: emb|Y14144.1| Length: 804 Number of Matches: 1

Related Information

Range 1: 93 to 521 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
726 bits(393)	0.0	418/430(97%)	1/430(0%)	Plus/Plus

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Query  61   GAAACCGGAGCTAATACCGGATAACACCGAAGACCGCATGGTCTTTGGTTGAAAGGCGGC  120
            ||| ||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct  153  GAAGCCGGAGCTAATACCGGATAACACCGAAGATCGCATGGTCTTTGGTTGAAAGGCGGC  212

Query  121  TTTGGCTGTCACTTACGGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTC  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  213  TTTGGCTGTCACTTACGGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTC  272

Query  181  ACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACA  240
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Sbjct  273  ACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACA  332

Query  241  CGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGA  300
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Sbjct  333  CGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGA  392

Query  301  CGGAtttttttttCGTGAGCGAAGAAGGTCTTCGGATTGTAAAGCTCTGTTGTTAGGGA  360
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Sbjct  393  CGGAGCAACGCCG-CGTGAGCGAAGAAGGTCTTCGGATTGTAAAGCTCTGTTGTTAGGGA  451

Query  361  AGAACAAGTACCGTTCGAAGAGGGCGGTACCGTGACGGTACCTAACGAGAAAGCCACGGC  420
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Sbjct  452  AGAACAAGTACCGTTCGAAGAGGGCGGTACCGTGACGGTACCTAACGAGAAAGCCACGGC  511

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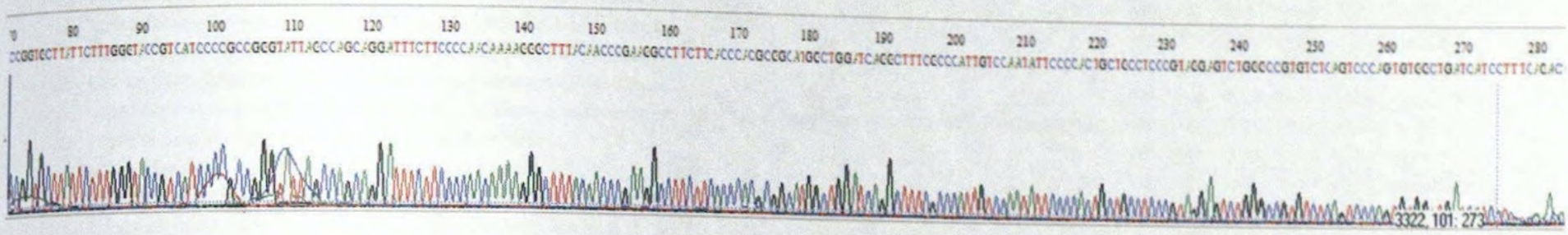
Placental tissue- P23 (b)



Fasta seq

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TCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGATCATCCT

BLAST report of sample 23 b

blast.ncbi.nlm.nih.gov/Blast.cgi

Uncultured Pseudoxanthomonas sp. clone ASC85 16S ribosomal RNA gene, partial sequence	425	425	100%	7e-116	100%	HQ912772.1
Pseudoxanthomonas taiwanensis partial 16S rRNA gene, strain L-bf-PMW-31.1	425	425	100%	7e-116	100%	FR774578.1
Stenotrophomonas sp. L-bf-PMW-31.3 partial 16S rRNA gene, strain L-bf-PMW-31.3	425	425	100%	7e-116	100%	FR774575.1
Xanthomonadales bacterium thermus-tsg1 16S ribosomal RNA gene, partial sequence	425	425	100%	7e-116	100%	HQ436530.1
Uncultured bacterium clone nbv256a02c1 16S ribosomal RNA gene, partial sequence	425	425	100%	7e-116	100%	HM812882.1
Uncultured bacterium clone nbv255h06c1 16S ribosomal RNA gene, partial sequence	425	425	100%	7e-116	100%	HM812878.1
Uncultured bacterium clone nbv255b10c1 16S ribosomal RNA gene, partial sequence	425	425	100%	7e-116	100%	HM812839.1
Uncultured bacterium clone nbw952e07c1 16S ribosomal RNA gene, partial sequence >gb|HM812745.1|Uncultured bacterium clone nbv253g06c1 16S ribo	425	425	100%	7e-116	100%	GQ040290.1
Uncultured compost bacterium partial 16S rRNA gene, clone PS3663	425	425	100%	7e-116	100%	FN667514.1
Uncultured compost bacterium partial 16S rRNA gene, clone PS3412	425	425	100%	7e-116	100%	FN667479.1
Uncultured bacterium clone nbw952b09c1 16S ribosomal RNA gene, partial sequence	425	425	100%	7e-116	100%	GQ040265.1
Pseudoxanthomonas taiwanensis 16S ribosomal RNA gene, partial sequence	425	425	100%	7e-116	100%	EU438976.1
Uncultured gamma proteobacterium gene for 16S rRNA, partial sequence, clone: COM-18	425	425	100%	7e-116	100%	AB451741.1
Uncultured Pseudoxanthomonas sp. clone LDC-8-c8 16S ribosomal RNA gene, partial sequence	425	425	100%	7e-116	100%	EU862350.1
Pseudoxanthomonas taiwanensis partial 16S rRNA gene, strain S22-48	425	425	100%	7e-116	100%	AM932275.1
Pseudoxanthomonas taiwanensis partial 16S rRNA gene, strain S22-31	425	425	100%	7e-116	100%	AM932267.1
Pseudoxanthomonas sp. NFC7-F12 16S ribosomal RNA gene, partial sequence	425	425	100%	7e-116	100%	EU250940.1
Pseudoxanthomonas sp. NFC7-A 16S ribosomal RNA gene, partial sequence	425	425	100%	7e-116	100%	EU250936.1
Pseudoxanthomonas sp. Ca7-1J03 16S ribosomal RNA gene, partial sequence	425	425	100%	7e-116	100%	EU177791.1
Pseudoxanthomonas sp. Ca7-5M04 16S ribosomal RNA gene, partial sequence	425	425	100%	7e-116	100%	EU177790.1

blast.ncbi.nlm.nih.gov/Blast.cgi#321156624

Pseudoxanthomonas taiwanensis partial 16S rRNA gene, strain L-bf-PMW-31.1

Sequence ID: emb|FR774578.1| Length: 954 Number of Matches: 1

Related Information

Range 1: 233 to 462 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
425 bits(230)	7e-116	230/230(100%)	0/230(0%)	Plus/Minus

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Query  61   GTATTAGCCAGCAGGATTTCTTCCCCAACAAAAGGGCTTTACAACCCGAAGGCCTTCTTC  120
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Sbjct  402  GTATTAGCCAGCAGGATTTCTTCCCCAACAAAAGGGCTTTACAACCCGAAGGCCTTCTTC  343

Query  121  ACCCACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTCCAATATTCCCCACTGCTGCC  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  342  ACCCACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTCCAATATTCCCCACTGCTGCC  283

Query  181  TCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGATCATCCT  230
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Sbjct  282  TCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGATCATCCT  233
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Stenotrophomonas sp. L-bf-PMW-31.3 partial 16S rRNA gene, strain L-bf-PMW-31.3

Sequence ID: emb|FR774575.1| Length: 954 Number of Matches: 1

Related Information

Range 1: 233 to 462 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
425 bits(230)	7e-116	230/230(100%)	0/230(0%)	Plus/Minus

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Query  61   GTATTAGCCAGCAGGATTTCTTCCCCAACAAAAGGGCTTTACAACCCGAAGGCCTTCTTC  120
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Query  181  TCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGATCATCCT  230
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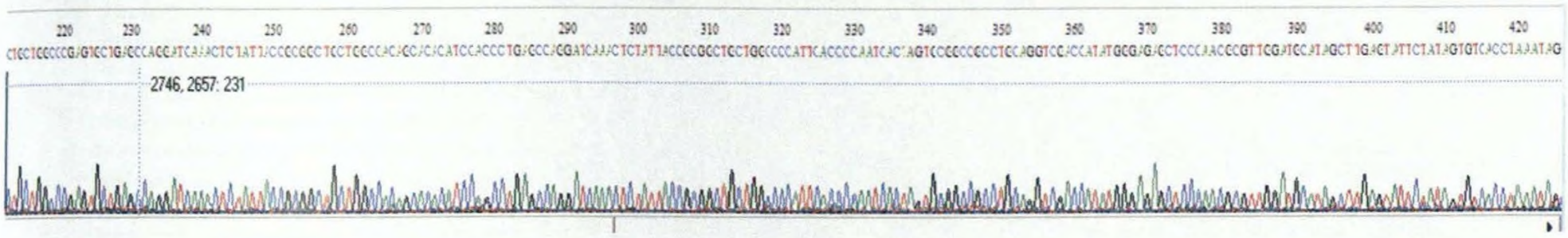
Placental Tissue- P26



Fasta seq

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CTAGTGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCAC
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BLAST report of sample P26

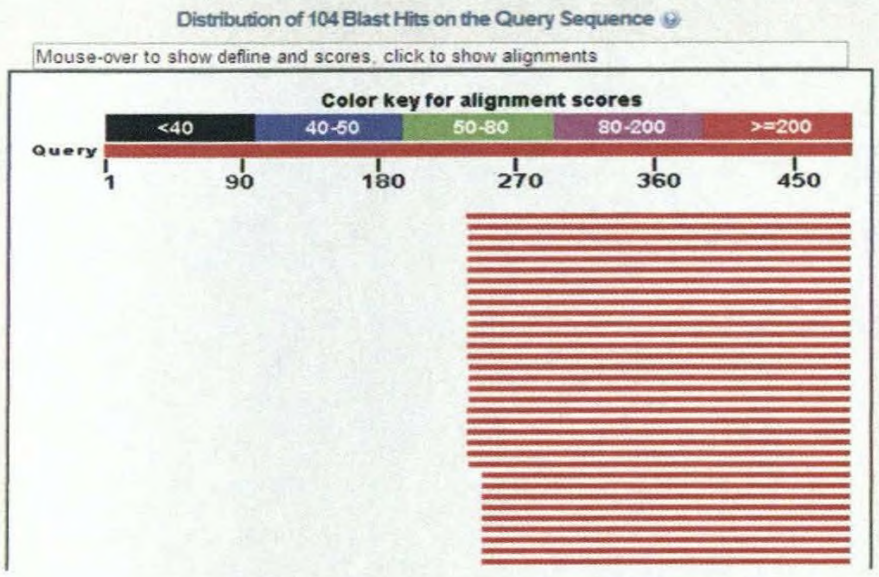
Placental tissue 26

RID M9S34MR301R (Expires on 04-10 13:53 pm)
Query ID lcl|234603
Description None
Molecule type nucleic acid
Query Length 488

Database Name nr
Description Nucleotide collection (nt)
Program BLASTN 2.2.29+ ▷ Citation

Other reports: ▷ Search Summary [Taxonomy reports] [Distance tree of results]

⊖ Graphic Summary



blast.ncbi.nlm.nih.gov/Blast.cgi

Alteromonas sp. 16S rRNA gene, isolate PRLIST1, partial	198	198	46%	2e-47	98%	Y15322.1
Cloning vector pSpark II, complete sequence	196	196	45%	6e-47	98%	GQ489185.1
Klebsiella pneumoniae class I integron dihydrofolate reductase (dhfrXII) and streptomycin 3"-adenylyltransferase (aadA2) genes, complete cds, and unknown g	196	196	44%	6e-47	99%	AF180731.1
Homo sapiens B120ind1 mRNA, 3'UTR, partial sequence	196	196	46%	6e-47	98%	AB032363.1
Paenibacillus polymyxa partial 16S rRNA gene, type strain DSM 36T, clone 11	195	195	46%	2e-46	97%	HG324074.1
Citrus reticulata microsatellite DNA, locus mCrCIR01B10, allele 01B10-tc(9)cc(1)tc(2)	195	195	46%	2e-46	97%	AM489733.1
Citrus reticulata microsatellite DNA, locus mCrCIR01E02, allele 01E02-ga(16)	193	193	46%	8e-46	97%	AM489735.1
Uncultured soil ascomycete partial 18S rRNA gene, clone r20-111	191	191	43%	3e-45	99%	AJ515922.1
Capsicum annuum cv. Bardenas mRNA for lipoxygenase (lox gene)	189	189	46%	1e-44	96%	FM164378.1
Uncultured soil basidiomycete partial 18S rRNA gene, clone r90-95	189	189	42%	1e-44	99%	AJ515939.1
Uncultured soil ascomycete partial 18S rRNA gene, clone r90-67	189	189	42%	1e-44	99%	AJ515932.1
Uncultured soil ascomycete partial 18S rRNA gene, clone r90-11	189	189	42%	1e-44	99%	AJ515925.1
Lathyrus sativus microsatellite DNA locus P2B9	185	185	42%	1e-43	99%	HF570153.1
Uncultured gamma proteobacterium partial 16S rRNA gene, clone ET3Rk66	185	185	42%	1e-43	99%	HG380017.1
Cloning vector pG-Sable, complete sequence	185	185	42%	1e-43	99%	KC537291.1
Uncultured Nitrospirae bacterium partial 16S rRNA gene, clone SC6-RK7	185	185	42%	1e-43	99%	HF584660.1
Cycas taitungensis microsatellite DNA, locus Cy-Tai SSR15	185	185	42%	1e-43	99%	FR744450.1
Camellia sinensis var. assamica microsatellite DNA locus Csin33	185	185	42%	1e-43	99%	HE802228.1
Uncultured bacterium partial 16S rRNA gene, clone ATB-AR-23621	185	185	42%	1e-43	99%	HE804882.1

Klebsiella pneumoniae class I integron dihydrofolate reductase (dhfrXII) and streptomycin 3"-adenylyltransferase (aadA2) genes, complete cds; and unknown gene

Sequence ID: gb|AF180731.1| Length: 2050 Number of Matches: 1

Range 1: 1942 to 2050 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
196 bits(106)	6e-47	108/109(99%)	0/109(0%)	Plus/Plus

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Sbjct  1942  TAATCACTAGTGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGAT  2001

Query  192   GCTTAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCA  240
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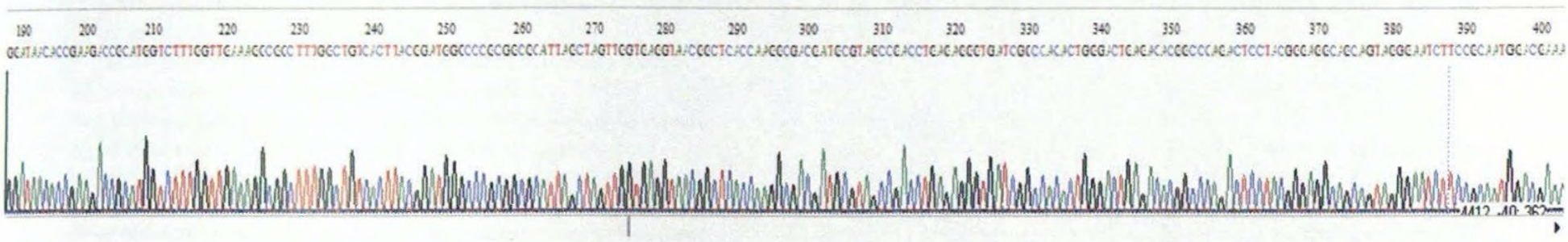
Related Information

Gene - associated gene details

Placental tissue- P30



Fasta seq

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BLAST report of sample P30

Placental tissue 30

RID M9T8CZKS01R (Expires on 04-10 14:13 pm)
Query ID lcl|45141
Description None
Molecule type nucleic acid
Query Length 613

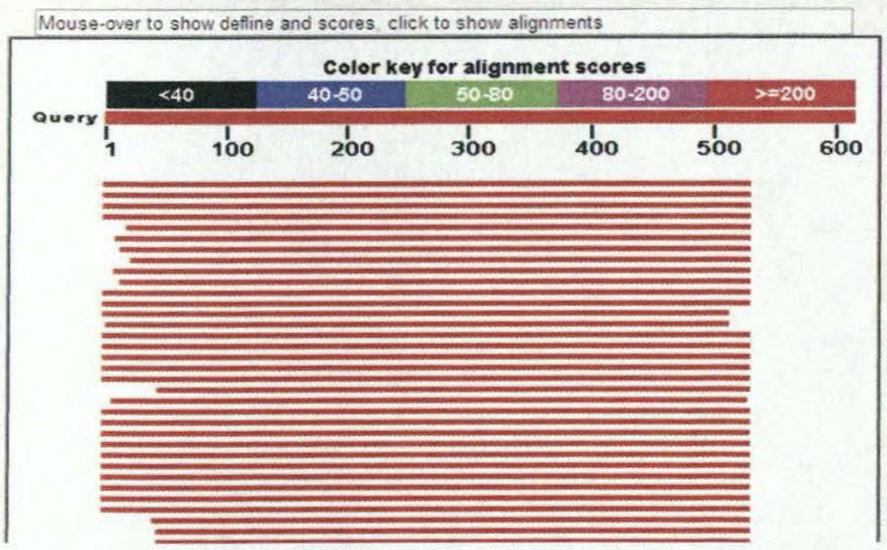
Database Name nr
Description Nucleotide collection (nt)
Program BLASTN 2.2.29+ ▷Citation

Other reports: ▷Search Summary [Taxonomy reports] [Distance tree of results]

⊖Graphic Summary

Distribution of 118 Blast Hits on the Query Sequence

Mouse-over to show defline and scores, click to show alignments



blast.ncbi.nlm.nih.gov/Blast.cgi

Uncultured bacteria clone F2-31 16S ribosomal RNA gene, partial sequence	736	736	100%	0.0	99%	AY096166.1
Bacillus sp. SSCA18 gene for 16S rRNA, partial sequence, strain: SSCA18	736	736	100%	0.0	99%	AB210934.1
Bacillus sp. SSCA19 gene for 16S rRNA, partial sequence, strain: SSCA19	736	736	100%	0.0	99%	AB210933.1
Bacillus sp. SSCA20 gene for 16S rRNA, partial sequence, strain: SSCA20	736	736	100%	0.0	99%	AB210932.1
Bacillus sp. partial 16S rRNA gene, isolate ISR04	736	736	100%	0.0	99%	Y14144.1
Uncultured Bacillaceae bacterium clone 2247.16S ribosomal RNA gene, partial sequence	730	730	100%	0.0	99%	KF503073.1
Anoxybacillus sp. PS2 16S ribosomal RNA gene, partial sequence	730	730	100%	0.0	99%	KF193531.1
Uncultured Bacillaceae bacterium clone 20G-e1 16S ribosomal RNA gene, partial sequence	730	730	100%	0.0	99%	KC918294.1
Uncultured Bacillus sp. clone 12CP-20 16S ribosomal RNA gene, partial sequence	730	730	100%	0.0	99%	KC918004.1
Uncultured Bacillaceae bacterium clone 18G-255 16S ribosomal RNA gene, partial sequence	730	730	100%	0.0	99%	KC917735.1
Uncultured Bacillaceae bacterium clone 16H-22 16S ribosomal RNA gene, partial sequence	730	730	100%	0.0	99%	KC917709.1
Uncultured Bacillaceae bacterium clone 16H-13 16S ribosomal RNA gene, partial sequence	730	730	100%	0.0	99%	KC917702.1
Uncultured Bacillaceae bacterium clone 16H-8 16S ribosomal RNA gene, partial sequence	730	730	100%	0.0	99%	KC917697.1
Uncultured Bacillaceae bacterium clone 16H-5 16S ribosomal RNA gene, partial sequence	730	730	100%	0.0	99%	KC917694.1
Uncultured Bacillaceae bacterium clone 16H-2 16S ribosomal RNA gene, partial sequence	730	730	100%	0.0	99%	KC917693.1
Uncultured Bacillaceae bacterium clone 16H-1 16S ribosomal RNA gene, partial sequence	730	730	100%	0.0	99%	KC917692.1

blast.ncbi.nlm.nih.gov/Blast.cgi#62701437

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Bacillus sp. SSCA18 gene for 16S rRNA, partial sequence, strain: SSCA18

Sequence ID: dbj|AB210934.1| Length: 528 Number of Matches: 1

Related Information

Range 1: 60 to 463 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
736 bits(398)	0.0	402/404(99%)	0/404(0%)	Plus/Plus

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Sbjct  60   GTTAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCCGTAAGATCGGGATAACTTC  119

Query  61   GGGAAACCGGAGCTAATACCGGATAACACCGAAGACCGCATGGTCTTTGGTTGAAAGGCG  120
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Sbjct  120  GGGAAACCGGAGCTAATACCGGATAACACCGAAGACCGCATGGTCTTTGGTTGAAAGGCG  179

Query  121  GCTTTGGCTGTCACTTACGGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGC  180
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Sbjct  180  GCTTTGGCTGTCACTTACGGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGC  239

Query  181  TCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGA  240
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Sbjct  240  TCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGA  299

Query  241  CACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCT  300
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Sbjct  300  CACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCT  359

Query  301  GACGGAGCAACGCCGCGTGAGCGAAGAAGGTCTTCGGATTGTAAAGCTCTGTTGTTAGGG  360
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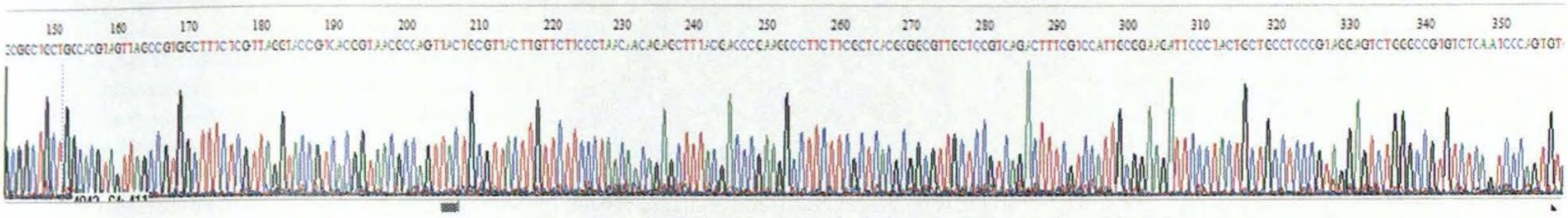
Placental tissue- P30 (b)



Fasta seq

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AAACTCTTAATCACCG

BLAST report of sample P30 b

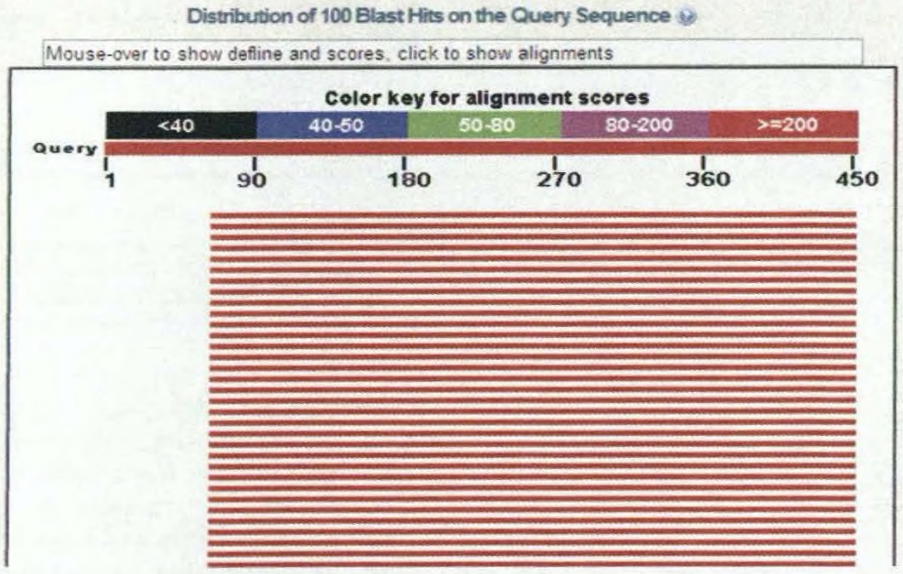
Placental tissue 30

RID M9U6MJEF01R (Expires on 04-10 14:29 pm)
Query ID lcl|20545
Description None
Molecule type nucleic acid
Query Length 452

Database Name nr
Description Nucleotide collection (nt)
Program BLASTN 2.2.29+ ▷Citation

Other reports: ▷Search Summary [Taxonomy reports] [Distance tree of results]

⊖Graphic Summary



blast.ncbi.nlm.nih.gov/Blast.cgi

Bacillus sp. KM2 16S ribosomal RNA gene, partial sequence	604	604	95%	1e-169	97%	GQ387040.1
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Bacillus thermoamylovorans strain IMAU80201 16S ribosomal RNA gene, partial sequence	604	604	95%	1e-169	97%	GU125619.1
Uncultured bacterium clone MC3F-8 16S ribosomal RNA gene, partial sequence	604	604	95%	1e-169	97%	GQ999971.1
Bacillus sp. 5.5LF 43T partial 16S rRNA gene, strain 5.5LF 43T	604	604	95%	1e-169	97%	FM958167.1
Bacterium C-TJ16 16S ribosomal RNA gene, partial sequence	604	604	95%	1e-169	97%	EU637623.1
Bacillus sp. 60LGv-1 gene for 16S rRNA, partial sequence	604	604	95%	1e-169	97%	AB375763.1
Bacillus sp. 50LAx-2 gene for 16S rRNA, partial sequence	604	604	95%	1e-169	97%	AB375753.1
Bacillus sp. 37LAv-3 gene for 16S rRNA, partial sequence	604	604	95%	1e-169	97%	AB375745.1
Bacillus circulans isolate 13 16S ribosomal RNA gene, partial sequence	604	604	95%	1e-169	97%	EU430989.1
Uncultured bacterium clone CS2_236 16S ribosomal RNA gene, partial sequence	604	604	95%	1e-169	97%	EF222018.1
Bacillus sp. B3 16S ribosomal RNA gene, partial sequence	604	604	95%	1e-169	97%	EU162044.1
Uncultured Bacilli bacterium clone MS090A1_B10 16S ribosomal RNA gene, partial sequence	604	604	95%	1e-169	97%	EF702816.1
Bacillus thermoamylovorans gene for 16S rRNA	604	604	95%	1e-169	97%	AB121094.1
Lactobacter thermoamylovorans partial 16S rRNA gene, isolate ISR01	604	604	95%	1e-169	97%	Y14141.1
B.circulans 16S rRNA gene, strain WSBC 20060	604	604	95%	1e-169	97%	Y13064.1
Bacillus thermoamylovorans partial 16S rRNA gene, strain 9-4AIA	601	601	95%	2e-168	96%	FN397520.1
Bacillus sp. N-11 gene for 16S ribosomal RNA, partial sequence	601	601	95%	2e-168	96%	AB618491.1

blast.ncbi.nlm.nih.gov/Blast.cgi#37196916

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Bacillus thermoamylovorans gene for 16S rRNA

Sequence ID: dbj|AB121094.1| Length: 1451 Number of Matches: 1

Related Information

Range 1: 154 to 516 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
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            | || || || || || ||||||||||||||||||||||||||||||||| |||||| ||
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blast.ncbi.nlm.nih.gov/Blast.cgi#194306121

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Sbjct  165  ATC  163
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Download GenBank Graphics ▼ Next ▲ Previous ⇞ Descriptions

Bacillus circulans isolate 13 16S ribosomal RNA gene, partial sequence

Sequence ID: gb|EU430989.1| Length: 1462 Number of Matches: 1

Related Information

Range 1: 133 to 495 GenBank Graphics ▼ Next Match ▲ Previous Match

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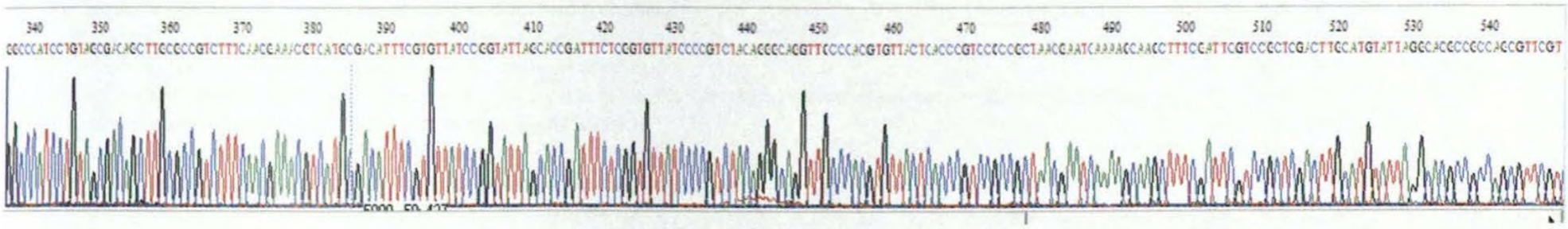
Placental tissue- P30 (c)



Fasta seq

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BLAST report of sample P30 c

Placental tissue 30

RID M9UYW6RV01R (Expires on 04-10 14:42 pm)
Query ID lcl|121939
Description None
Molecule type nucleic acid
Query Length 615

Database Name nr
Description Nucleotide collection (nt)
Program BLASTN 2.2.29+ ▷Citation

Other reports: ▷Search Summary [Taxonomy reports] [Distance tree of results]

⊖Graphic Summary

Distribution of 107 Blast Hits on the Query Sequence

Mouse-over to show defline and scores, click to show alignments

blast.ncbi.nlm.nih.gov/Blast.cgi

Uncultured bacterium clone WB55 16S ribosomal RNA gene, partial sequence	974	974	86%	0.0	99%	JX133633.1
Uncultured bacterium clone S15 16S ribosomal RNA gene, partial sequence	974	974	86%	0.0	99%	JX133367.1
Uncultured Anoxybacillus sp. clone KAS-R35 16S ribosomal RNA gene, partial sequence	974	974	86%	0.0	99%	JX110482.1
Uncultured Anoxybacillus sp. clone KAS-R30 16S ribosomal RNA gene, partial sequence	974	974	86%	0.0	99%	JX110477.1
Uncultured Anoxybacillus sp. clone KAS-R1 16S ribosomal RNA gene, partial sequence	974	974	86%	0.0	99%	JX110448.1
Uncultured bacterium gene for 16S rRNA, partial sequence, clone: In_stim_milk-Clone12	974	974	86%	0.0	99%	AB356013.1
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Anoxybacillus flavithermus clone LK4 16S ribosomal RNA gene, partial sequence	972	972	86%	0.0	99%	EU810669.1
Bacterium SR55-4 16S ribosomal RNA gene, partial sequence	970	970	86%	0.0	99%	KF279370.1
Anoxybacillus flavithermus strain A13 16S ribosomal RNA gene, partial sequence	970	970	86%	0.0	99%	KC310464.1
Anoxybacillus kestanbolensis strain A1 16S ribosomal RNA gene, partial sequence	970	970	86%	0.0	99%	KC310452.1
Anoxybacillus flavithermus strain SR55-3 16S ribosomal RNA gene, partial sequence	968	968	86%	0.0	99%	KF279369.1
Uncultured bacterium clone L2B-B12 16S ribosomal RNA gene, partial sequence	968	968	86%	0.0	99%	JQ894879.1
Uncultured Anoxybacillus sp. clone KAS-R9 16S ribosomal RNA gene, partial sequence	968	968	86%	0.0	99%	JX110458.1
Uncultured bacterium clone IC48 16S ribosomal RNA gene, partial sequence	968	968	86%	0.0	99%	GQ359988.1
Uncultured bacterium clone nbf246b10 16S ribosomal RNA gene, partial sequence	968	968	86%	0.0	99%	EU540152.1
Uncultured bacterium gene for 16S rRNA, partial sequence, clone: In_stim_milk-Clone58	968	968	86%	0.0	99%	AB356009.1
Uncultured bacterium clone pt59 16S ribosomal RNA gene, partial sequence	968	968	86%	0.0	99%	DQ890423.1
Unidentified proteobacterium gene for 16S ribosomal RNA, partial sequence, clone: BD5-12	968	968	86%	0.0	99%	AB015568.1
Uncultured bacterium clone 7-36 16S ribosomal RNA gene, partial sequence	966	966	86%	0.0	99%	KC170395.1
Unidentified marine bacterioplankton clone P2-1-1B_27 16S ribosomal RNA gene, partial sequence	966	966	86%	0.0	99%	KC000800.1
Anoxybacillus kestanbolinensis strain K1 16S ribosomal RNA gene, partial sequence	965	965	86%	0.0	99%	AY248709.1

blast.ncbi.nlm.nih.gov/Blast.cgi#545793783

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Anoxybacillus flavithermus strain SR55-3 16S ribosomal RNA gene, partial sequence

Sequence ID: gb|KF279366.1| Length: 1513 Number of Matches: 1

Related Information

Range 1: 1 to 530 GenBank Graphics

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Sbjct  470  AGTTACTGCGTTACTTGTTCTTCCCTAACAACAGAGCTTTACGACCCGAAGGCCTTCTTC  411

Query  121  GCTCACGCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGATTCCCTACTGCTGCC  180
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Query  181  TCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGG  240
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Sbjct  350  TCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGG  291

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Sbjct  230  ATCCTGTAGCGACAGCTTGCGCCGTCTTTCAACGAAACGTCATGCGACATTTCGTGTTAT  171

Query  361  CCGGTATTAGCACCGATTTCTCGGTGTTATCCCCGTCTACAGGGCAGGTTGCCCACGTGT  420
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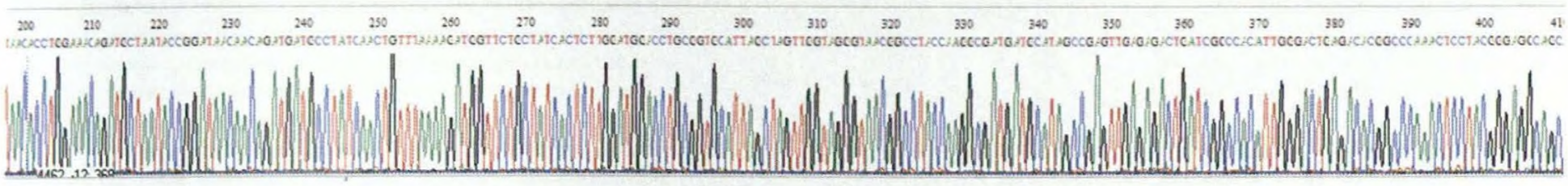
Placental tissue- P34



Fasta seq

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BLAST report of sample P34

Placental tissue 34

RID M9W8A0UE01R (Expires on 04-10 15:04 pm)
Query ID lcl|52611
Description None
Molecule type nucleic acid
Query Length 473

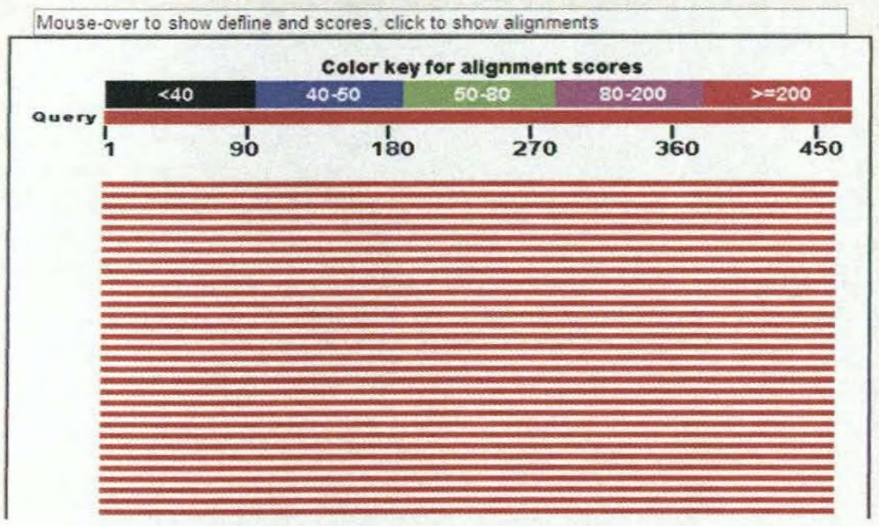
Database Name nr
Description Nucleotide collection (nt)
Program BLASTN 2.2.29+ ▷Citation

Other reports: ▷Search Summary [Taxonomy reports] [Distance tree of results]

⊖Graphic Summary

Distribution of 100 Blast Hits on the Query Sequence

Mouse-over to show defline and scores, click to show alignments



blast.ncbi.nlm.nih.gov/Blast.cgi

Uncultured bacterium clone ncd509f07c1 16S ribosomal RNA gene, partial sequence >gb|JF181373.1|Uncultured bacterium clone ncd2093g05c1 16S ribosom	822	822	98%	0.0	99%	HM275684.1
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Uncultured bacterium isolate CH96J00003 16S ribosomal RNA gene, partial sequence

Sequence ID: gb|EF365407.1| Length: 645 Number of Matches: 1

Related Information

Range 1: 33 to 496 GenBank Graphics ▼ Next Match ▲ Previous Match

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Query  121  AACTGTTTAAAAGATGGTTCTGCTATCACTCTTGGATGGACCTGCGGTGCATTAGCTAGT  180
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Sbjct  153  AACTGTTTAAAAGATGGTTCTGCTATCACTCTTGGATGGACCTGCGGTGCATTAGCTAGT  212

Query  181  TGGTAGGGTAACGGCCTACCAAGGCGATGATGCATAGCCGAGTTGAGAGACTGATCGGCC  240
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Sbjct  213  TGGTAGGGTAACGGCCTACCAAGGCGATGATGCATAGCCGAGTTGAGAGACTGATCGGCC  272

Query  241  ACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCAC  300
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Sbjct  273  ACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCAC  332

Query  301  AATGGACttttttCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAA  360
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Query  421  ATTAGAAAGTCACGGCTAACTACGTGCCAGCAGCCGCGGTAATAA  465
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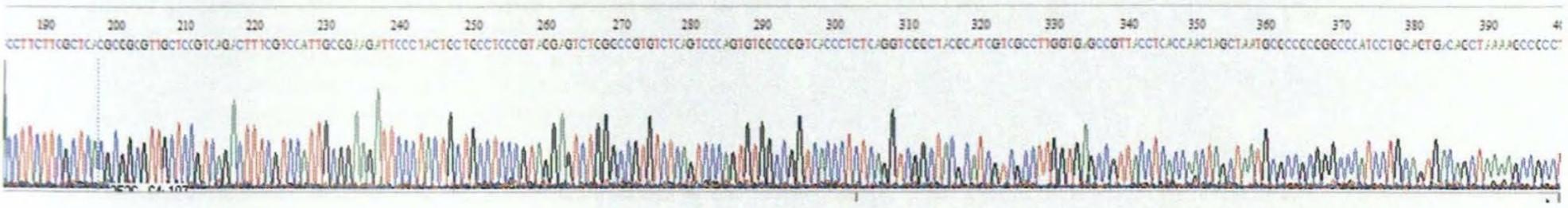
Placental tissue- P36



Fasta seq

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CGCATCGTCGCCTTGGTGAGCCGTTACCTCACCAACTAGCTAATGCGCCGCGGGCCCATCCTGCAGTGACAGCTAAAAGCCG
CCTTTCAACCGAAAACCATGCGGTTTTCGGTGTTATCCGGTATTAGCTCCGGTTTCCCGAAGTTATCCCAGTCTGCAAGGCAG
GTTGCCCACGTGTTACTCAC

BLAST report of sample P36

Placental tissue 36

RID M9TZ3PUF01R (Expires on 04-10 14:25 pm)
Query ID lcl|164179
Description None
Molecule type nucleic acid
Query Length 351

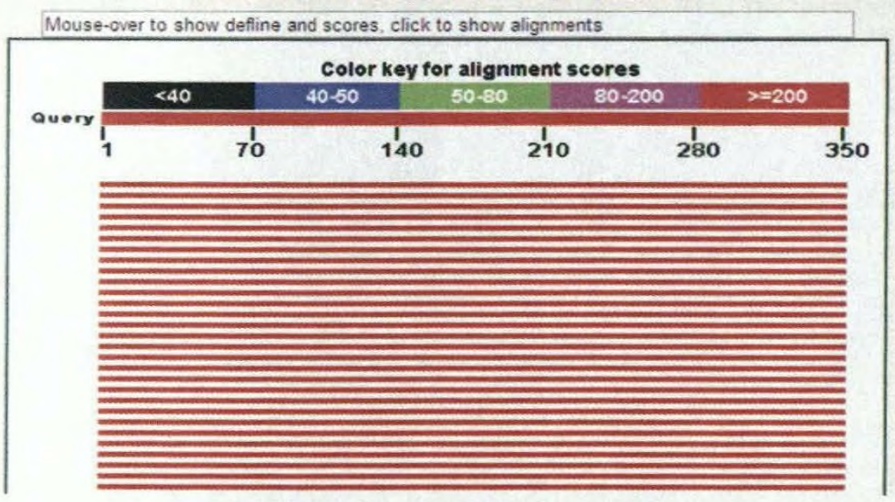
Database Name nr
Description Nucleotide collection (nt)
Program BLASTN 2.2.29+ ▷Citation

Other reports: ▷Search Summary [Taxonomy reports] [Distance tree of results]

⊖Graphic Summary

Distribution of 100 Blast Hits on the Query Sequence

Mouse-over to show defline and scores, click to show alignments



blast.ncbi.nlm.nih.gov/Blast.cgi

Geobacillus sp. T38 16S ribosomal RNA gene, partial sequence	638	638	100%	1e-179	99%	DQ842094.1
Bacillus sp. IS-2004 16S ribosomal RNA gene, partial sequence	638	638	100%	1e-179	99%	AY639816.1
Bacillus sp. E53-10 16S ribosomal RNA gene, complete sequence	638	638	100%	1e-179	99%	AY583458.1
Bacillus sp. BS1 16S ribosomal RNA gene, partial sequence	638	638	100%	1e-179	99%	AY566220.1
Bacillus sp. JH1 16S ribosomal RNA gene, partial sequence	638	638	100%	1e-179	99%	AY554279.1
Bacillus sp. N-4-55-3 gene for 16S rRNA, partial sequence	638	638	100%	1e-179	99%	AB190021.1
Bacillus sp. N-0-55-9 gene for 16S rRNA, partial sequence	638	638	100%	1e-179	99%	AB190007.1
Bacillus sp. SSCA48 gene for 16S rRNA, partial sequence, strain: SSCA48	638	638	100%	1e-179	99%	AB210904.1
Bacillus sp. S-0-55-5 gene for 16S rRNA, partial sequence	638	638	100%	1e-179	99%	AB190136.1
Geobacillus pallidus gene for 16S rRNA, partial sequence, strain:D-0-55-3	638	638	100%	1e-179	99%	AB190088.1
Uncultured Geobacillus sp. partial 16S rRNA gene, isolate Td	638	638	100%	1e-179	99%	AJ564615.1
Uncultured Geobacillus sp. partial 16S rRNA gene, isolate F16	638	638	100%	1e-179	99%	AJ564609.1
Bacillus pallidus strain R-7748 16S ribosomal RNA gene, partial sequence	638	638	100%	1e-179	99%	AY373321.1
Bacillus sp. IZ5 gene for 16S ribosomal RNA, partial sequence	638	638	100%	1e-179	99%	AB089235.1

Bacillus sp. SSCA48 gene for 16S rRNA, partial sequence, strain: SSCA48

Sequence ID: dbj|AB210904.1| Length: 495 Number of Matches: 1

Related Information

Range 1: 85 to 435 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
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Sbjct  435  TTCTTCCCTGACAACAGAGCTTTACGATCCGAAGACCTTCTTCGCTCACGCGGCGTTGCT  376

Query  61   CCGTCAGACTTTCGTCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGG  120
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Sbjct  375  CCGTCAGACTTTCGTCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGG  316

Query  121  CCGTGTCTCAGTCCCAGTGTGGCCGGTCACCCTCTCAGGTCGGCTACGCATCGTCGCCTT  180
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Sbjct  315  CCGTGTCTCAGTCCCAGTGTGGCCGGTCACCCTCTCAGGTCGGCTACGCATCGTCGCCTT  256

Query  181  GGTGAGCCGTTACCTCACCAACTAGCTAATGCGCCGCGGGCCCATCCTGCAGTGACAGCT  240
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Sbjct  255  GGTGAGCCGTTACCTCACCAACTAGCTAATGCGCCGCGGGCCCATCCTGCAGTGACAGCT  196

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Sbjct  195  AAAAGCCGCCTTTCAACCGAAAACCATGCGGTTTTCGGTGTTATCCGGTATTAGCTCCGG  136

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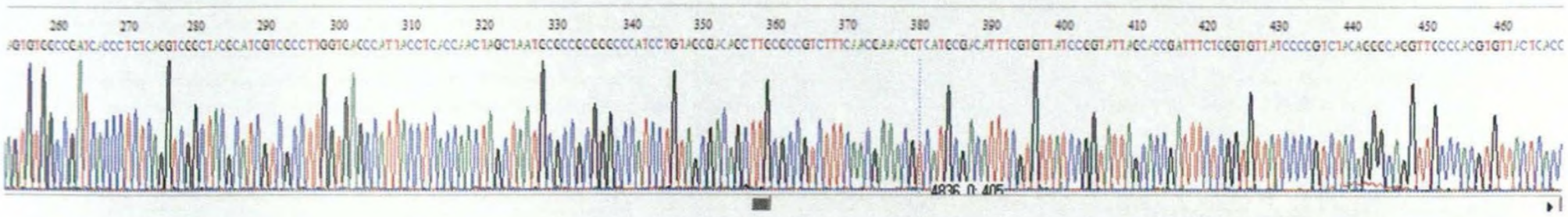
Placental tissue- P36 (b)



Fasta seq

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GTCGCCGCCCTCGGTATTAGCACCGGATTTTTTTGGTGTTATCCCCCGTCTTCAGGGGCAGGGTTGCCCCAGTGTTTACTCCCC
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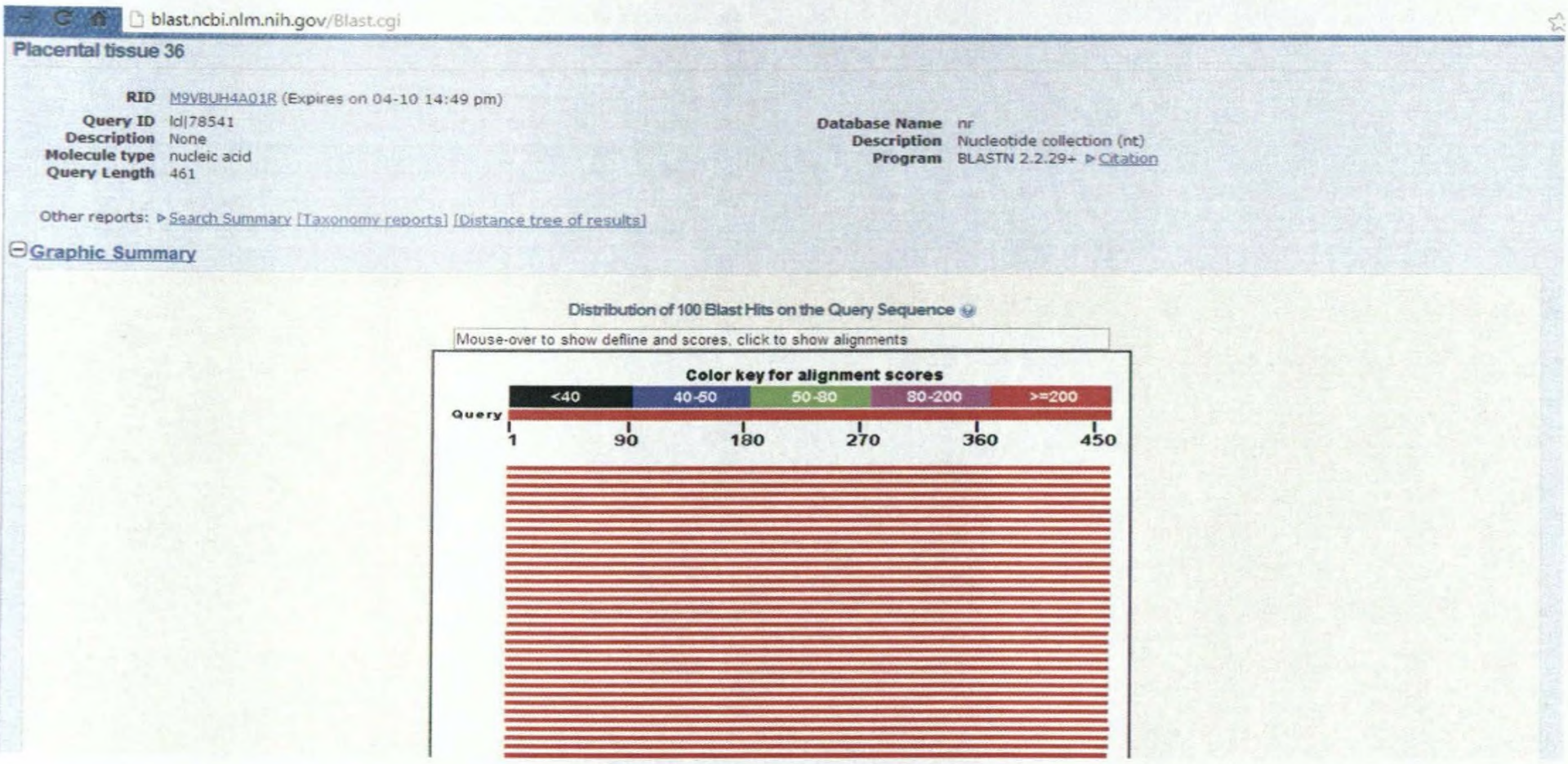
BLAST report of P36 b



Anoxybacillus flavithermus strain A13 16S ribosomal RNA gene, partial sequence	846	846	100%	0.0	99%	KC310464.1
Anoxybacillus kestanbolensis strain A1 16S ribosomal RNA gene, partial sequence	846	846	100%	0.0	99%	KC310452.1
Unidentified marine bacterioplankton clone P2-1-1B_27 16S ribosomal RNA gene, partial sequence	846	846	100%	0.0	99%	KC000800.1
Uncultured bacterium clone WB55 16S ribosomal RNA gene, partial sequence	846	846	100%	0.0	99%	JX133633.1
Uncultured bacterium clone S15 16S ribosomal RNA gene, partial sequence	846	846	100%	0.0	99%	JX133367.1
Uncultured Anoxybacillus sp. clone KAS-R35 16S ribosomal RNA gene, partial sequence	846	846	100%	0.0	99%	JX110482.1
Uncultured Anoxybacillus sp. clone KAS-R30 16S ribosomal RNA gene, partial sequence	846	846	100%	0.0	99%	JX110477.1
Uncultured Anoxybacillus sp. clone KAS-R1 16S ribosomal RNA gene, partial sequence	846	846	100%	0.0	99%	JX110448.1
Uncultured organism clone ELU0097-T49-S-NI_000209 small subunit ribosomal RNA gene, partial sequence	846	846	100%	0.0	99%	HQ783665.1
Uncultured organism clone ELU0097-T49-S-NI_000073 small subunit ribosomal RNA gene, partial sequence	846	846	100%	0.0	99%	HQ783529.1
Uncultured organism clone ELU0092-T15-S-NI_000522 small subunit ribosomal RNA gene, partial sequence	846	846	100%	0.0	99%	HQ781957.1
Uncultured compost bacterium gene for 16S ribosomal RNA, partial sequence, clone: 272-2	846	846	100%	0.0	99%	AB555715.1
Anoxybacillus flavithermus clone LK4 16S ribosomal RNA gene, partial sequence	846	846	100%	0.0	99%	EU816589.1
Uncultured bacterium gene for 16S rRNA, partial sequence, clone: In_skim_milk-Clone12	846	846	100%	0.0	99%	AB356013.1
Uncultured bacterium gene for 16S rRNA, partial sequence, clone: In_skim_milk-Clone61 >dbjlAB356097.1|Uncultured bacterium gene for 16S rRNA, partial ser	846	846	100%	0.0	99%	AB356062.1
Anoxybacillus flavithermus strain C 16S ribosomal RNA gene, partial sequence	846	846	100%	0.0	99%	AY643748.1
Bacterium SR55-4 16S ribosomal RNA gene, partial sequence	845	845	99%	0.0	99%	KF279379.1
Uncultured bacterium clone L2B-B12 16S ribosomal RNA gene, partial sequence	845	845	99%	0.0	99%	JQ894876.1
Anoxybacillus flavithermus strain Qa4 16S ribosomal RNA gene, partial sequence	845	845	99%	0.0	99%	KC252986.1
Uncultured Firmicutes bacterium partial 16S rRNA gene, clone MP806-1	845	845	99%	0.0	99%	HE583206.1

Anoxybacillus flavithermus strain A13 16S ribosomal RNA gene, partial sequence

Sequence ID: gb|KC310464.1| Length: 1428 Number of Matches: 1

Range 1: 25 to 485 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
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Sbjct  425  GGCCTTCTTCGCTCACGCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGATTCCC  366

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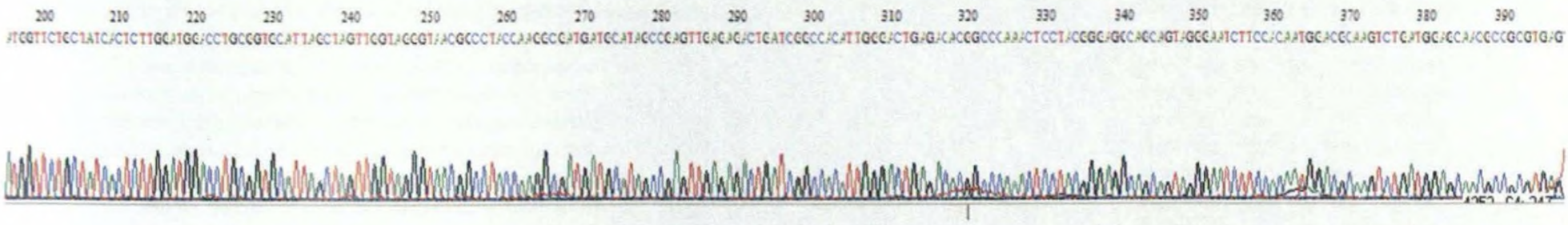
Related Information

Placental tissue- P54



Fasta seq

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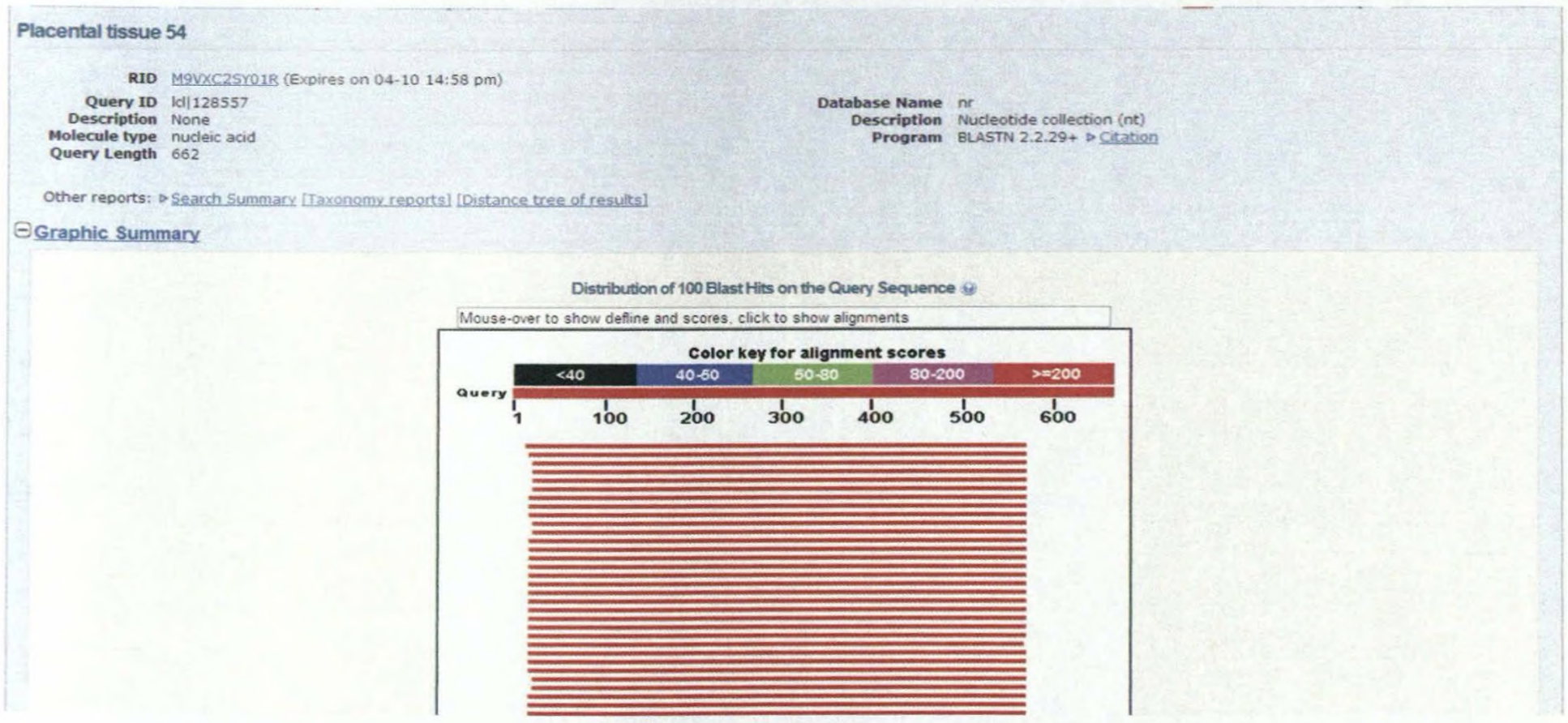
BLAST report of P54



Uncultured Firmicutes bacterium clone F12_3B_FF 16S ribosomal RNA gene, partial sequence	1005	1005	83%	0.0	99%	EF682864.1
Uncultured Firmicutes bacterium gene for 16S ribosomal RNA, partial sequence, clone: AN004	1002	1002	82%	0.0	99%	AB809957.1
Uncultured bacterium clone 16slp100-3e08.p1k 16S ribosomal RNA gene, partial sequence	1002	1002	82%	0.0	99%	GQ156700.1
Uncultured bacterium clone 16slp100-3c09.p1k 16S ribosomal RNA gene, partial sequence	1002	1002	82%	0.0	99%	GQ156699.1
Uncultured Lactobacillus sp. clone EHFS1_S05c 16S ribosomal RNA gene, partial sequence	1002	1002	82%	0.0	99%	EU071482.1
Uncultured Firmicutes bacterium clone F11_3A_FL 16S ribosomal RNA gene, partial sequence	1002	1002	82%	0.0	99%	EF682871.1
Uncultured bacterium clone Z71 16S ribosomal RNA gene, partial sequence	1002	1002	82%	0.0	99%	DQ666105.1
Uncultured bacterium clone C15 16S ribosomal RNA gene, partial sequence	1002	1002	82%	0.0	99%	DQ666101.1
Uncultured bacterium clone C9 16S ribosomal RNA gene, partial sequence	1002	1002	82%	0.0	99%	DQ666100.1
Lactobacillus iners clone FX43-4 16S ribosomal RNA gene, partial sequence	1002	1002	82%	0.0	99%	AY283270.1
Lactobacillus iners clone FX9-5 16S ribosomal RNA gene, partial sequence	1002	1002	82%	0.0	99%	AY283269.1
Uncultured bacterium clone rRNA420 16S ribosomal RNA gene, partial sequence	1002	1002	82%	0.0	99%	AY959193.1
Uncultured bacterium clone rRNA393 16S ribosomal RNA gene, partial sequence	1002	1002	82%	0.0	99%	AY959166.1
Uncultured bacterium clone rRNA390 16S ribosomal RNA gene, partial sequence	1002	1002	82%	0.0	99%	AY959163.1
Uncultured bacterium clone rRNA383 16S ribosomal RNA gene, partial sequence	1002	1002	82%	0.0	99%	AY959156.1
Uncultured bacterium clone rRNA342 16S ribosomal RNA gene, partial sequence	1002	1002	82%	0.0	99%	AY959115.1
Uncultured bacterium clone rRNA327 16S ribosomal RNA gene, partial sequence	1002	1002	82%	0.0	99%	AY959100.1

Lactobacillus iners clone FX43-4 16S ribosomal RNA gene, partial sequence

Sequence ID: gb|AY283270.1| Length: 937 Number of Matches: 1

Related Information

Range 1: 1 to 545 GenBank Graphics

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Query  84   AAGATCGGAGTGCTTGCACTCTGTGAAACAAGATACAGGCTAGCGGCGGACGGGTGAGTA  143
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Sbjct  61   AAGATCGGAGTGCTTGCACTCTGTGAAACAAGATACAGGCTAGCGGCGGACGGGTGAGTA  120

Query  144  ACACGTGGGTAACCTGCCCAAGAGATCGGGATAACACCTGGAAACAGATGCTAATACCGG  203
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Query  204  ATAACAACAGATGATGCCTATCAACTGTTTAAAAGATGGTTCTGCTATCACTCTTGGATG  263
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Sbjct  181  ATAACAACAGATGATGCCTATCAACTGTTTAAAAGATGGTTCTGCTATCACTCTTGGATG  240

Query  264  GACCTGCGGTGCATTAGCTAGTTGGTAGGGTAACGGCCTACCAAGGCGATGATGCATAGC  323
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Sbjct  361  GCAGCAGTAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTG  420

Query  444  AAGAAGGGTTTCGGCTCGTAAAGCTCTGTTGTTGGTGAAGAAGGACAGGGGTAGTAACTG  503
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Sbjct  541  TAATA  545
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7.2 TEST REPORT OF AGILENT HIGH SENSITIVITY DNA ASSAY

Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE04103941_2013-05-27_02-38-15.xad

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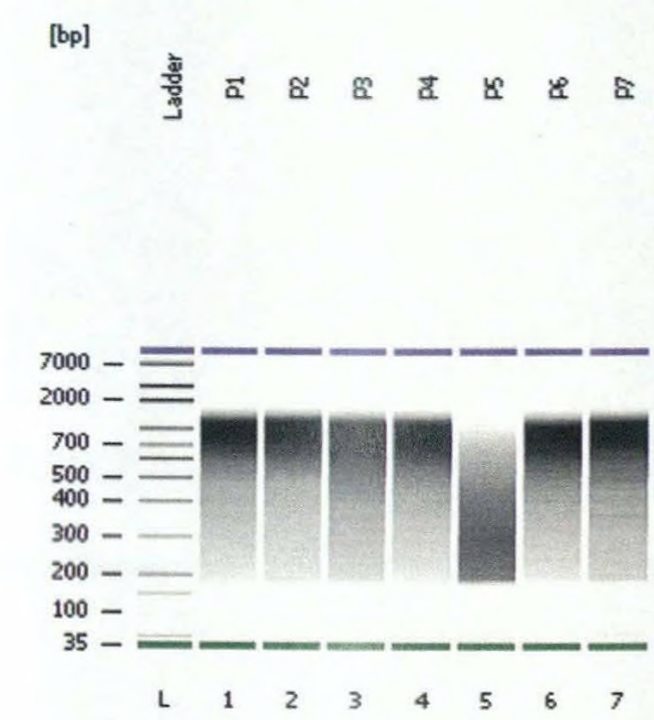
Modified: 5/27/2013 3:58:57 AM

Electrophoresis File Run Summary



Instrument Information:

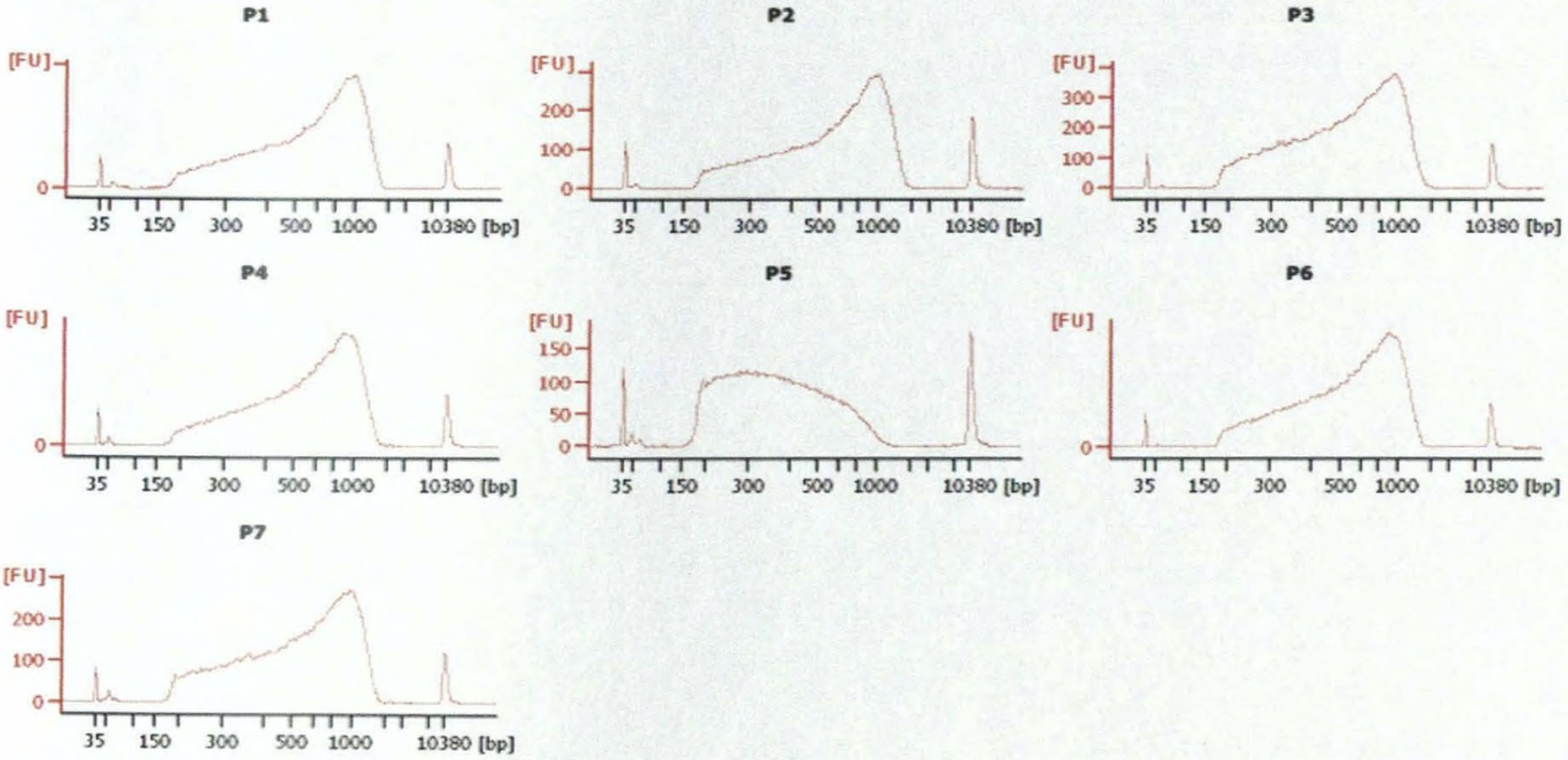
Instrument Name: DE04103941 Firmware: C.01.069
Serial#: DE04103941 Type: G2939A

Assay Information:

Assay Origin Path: C:\Program Files\Agilent\2100 bioanalyzer\2100 expert\assays\dsDNA\High Sensitivity DNA.xsy
Assay Class: High Sensitivity DNA Assay
Version: 1.03
Assay Comments: Copyright © 2003-2010 Agilent Technologies

Chip Information:

Chip Lot #:
Reagent Kit Lot #:
Chip Comments:



Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE04103941_2013-05-27_02-38-15.xad

Created: 5/27/2013 2:38:15 AM
Modified: 5/27/2013 3:58:57 AM

Electrophoresis File Run Summary (Chip Summary)

Sample Name	Sample Comment	Rest. Digest	Status	Observation	Result Label	Result Color
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P2		☐	✔			
P3		☐	✔			
P4		☐	✔			
P5		☐	✔			
P6		☐	✔			
P7		☐	✔			
Blank		☐				
Blank		☐				
Blank		☐				
Blank		☐				
Ladder		☐	✔			

Chip Lot # **Reagent Kit Lot #**

Chip Comments :

Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE04103941_2013-05-27_02-38-15.xad

Created: 5/27/2013 2:38:15 AM
Modified: 5/27/2013 3:58:57 AM

Electrophoresis Assay Details

General Analysis Settings

Number of Available Sample and Ladder Wells (Max.) : 12
Minimum Visible Range [s] : 32
Maximum Visible Range [s] . 138
Start Analysis Time Range [s] : 33
End Analysis Time Range [s] : 137.5
Ladder Concentration [pg/µl] : 1950
Uses Standard Area for Ladder Fragments
Lower Marker Concentration [pg/µl] : 125
Upper Marker Concentration [pg/µl] : 75
Used Upper Marker for Quantitation
Standard Curve Fit is Point to Point
Show Data Aligned to Lower and Upper Marker

Integrator Settings

Integration Start Time [s] : 33.05
Integration End Time [s] : 137
Slope Threshold : 0.8
Height Threshold [FU] : 5
Area Threshold : 0.1
Width Threshold [s] : 0.6
Baseline Plateau [s] : 0.5

Filter Settings

Filter Width [s] : 0.5
Polynomial Order : 4

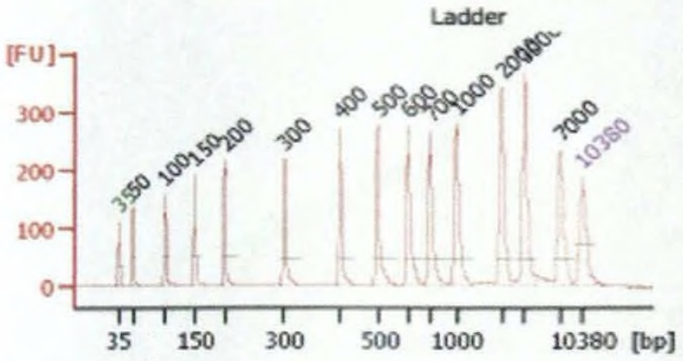
Ladder

Ladder Peak	Size	Area
1	35	160
2	50	210
3	100	208
4	150	221
5	200	242
6	300	270
7	400	305
8	500	306
9	600	336
10	700	321
11	1000	366
12	2000	413
13	3000	411
14	7000	400
15	10380	214

Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE04103941_2013-05-27_02-38-15.xad

Created: 5/27/2013 2:38:15 AM
Modified: 5/27/2013 3:58:57 AM

Electropherogram Summary



Overall Results for Ladder

Noise: 0.5

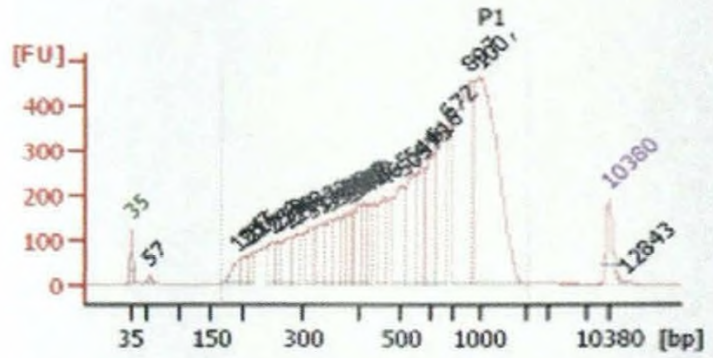
Peak table for Ladder

Peak	Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1	35	125.00	5,411.3	Lower Marker
2	50	150.00	4,545.5	Ladder Peak
3	100	150.00	2,272.7	Ladder Peak
4	150	150.00	1,515.2	Ladder Peak
5	200	150.00	1,136.4	Ladder Peak
6	300	150.00	757.6	Ladder Peak
7	400	150.00	568.2	Ladder Peak
8	500	150.00	454.5	Ladder Peak
9	600	150.00	378.8	Ladder Peak
10	700	150.00	324.7	Ladder Peak
11	1,000	150.00	227.3	Ladder Peak
12	2,000	150.00	113.6	Ladder Peak
13	3,000	150.00	75.8	Ladder Peak
14	7,000	150.00	32.5	Ladder Peak
15	10,380	75.00	10.9	Upper Marker

Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE04103941_2013-05-27_02-38-15.xad

Created: 5/27/2013 2:38:15 AM
Modified: 5/27/2013 3:58:57 AM

Electropherogram Summary Continued ...



Overall Results for sample 1 : P1

Number of peaks found:	27
Noise:	0.5
Corr. Area 1:	10,195.9

Region table for sample 1 : P1

From [bp]	To [bp]	Corr. Area	% of Total	Average Size [bp]	Size distribution in CV [%]	Conc. [pg/µl]	Molarity [pmol/l]	Color
169	2,117	10,195.9	99	681	51.8	5,722.87	18,803.6	

Peak table for sample 1 : P1

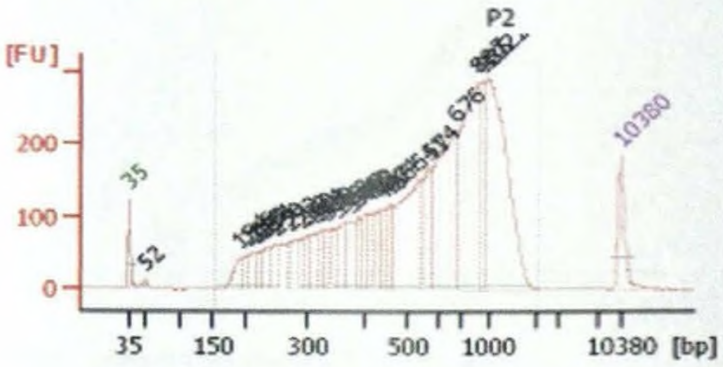
Peak	Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1	35	125.00	5,411.3	Lower Marker
2	57	42.66	1,143.8	
3	194	102.72	801.4	
4	207	87.06	636.6	
5	216	71.70	502.1	
6	250	134.03	811.7	
7	259	76.64	447.7	
8	278	147.22	801.6	
9	290	133.31	697.2	
10	313	140.10	679.2	
11	333	140.72	639.9	
12	348	136.63	594.5	
13	361	132.91	558.2	
14	370	83.89	343.4	
15	378	83.24	333.4	
16	388	83.70	327.2	
17	399	114.47	434.8	
18	408	104.62	388.1	
19	422	123.79	444.5	
20	465	208.46	679.9	
21	509	280.89	836.2	
22	544	248.56	692.9	
23	575	205.51	541.8	
24	616	254.62	625.9	
25	672	338.85	764.0	
26	897	643.51	1,087.0	

Peak	Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
27	1,007	882.93	1,328.3	
28	10,380	75.00	10.9	Upper Marker
29	12,843	0.00	0.0	

Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE04103941_2013-05-27_02-38-15.xad

Created: 5/27/2013 2:38:15 AM
Modified: 5/27/2013 3:58:57 AM

Electropherogram Summary Continued ...



Overall Results for sample 2 : P2

Number of peaks found:	28
Noise:	0.4
Corr. Area 1:	6,355.1

Region table for sample 2 : P2

From [bp]	To [bp]	Corr. Area	% of Total	Average Size [bp]	Size distribution in CV [%]	Conc. [pg/µl]	Molarity [pmol/l]	Color
156	2,169	6,355.1	99	681	52.3	3,830.00	12,772.6	

Peak table for sample 2 : P2

Peak	Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1	35	125.00	5,411.3	Lower Marker
2	52	25.36	737.4	
3	194	88.90	694.9	
4	206	48.17	353.4	
5	218	57.60	401.1	
6	223	46.88	317.9	
7	234	87.85	567.7	
8	250	82.68	500.7	
9	272	54.90	305.4	
10	290	57.35	299.8	
11	300	57.87	292.5	
12	314	82.34	397.7	
13	321	64.67	305.5	
14	332	68.57	313.4	
15	349	71.46	310.5	
16	362	94.15	394.0	
17	374	107.07	433.5	
18	387	62.49	244.7	
19	400	77.35	292.7	
20	408	57.01	211.8	
21	437	70.98	246.0	
22	456	62.70	208.5	
23	464	69.86	228.1	
24	541	342.49	959.4	
25	574	123.52	326.2	
26	676	445.56	999.0	
27	887	479.01	818.6	
28	935	127.70	207.0	
29	1,022	561.65	832.8	
30	10,380	75.00	10.9	Upper Marker

Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE04103941_2013-05-27_02-38-15.xad

Created: 5/27/2013 2:38:15 AM
Modified: 5/27/2013 3:58:57 AM

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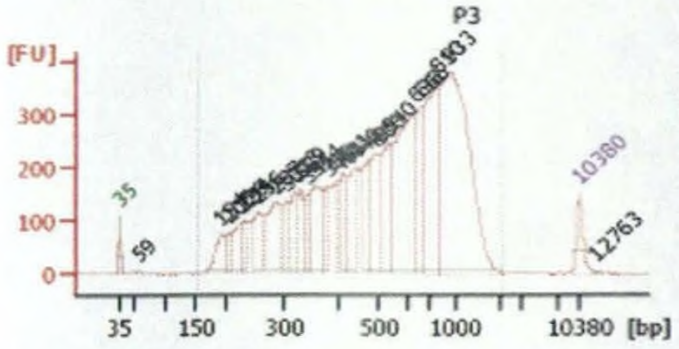

Overall Results for sample 3 : P3

Number of peaks found:	24
Noise:	0.6
Corr. Area 1:	9,858.5

Region table for sample 3 : P3

From [bp]	To [bp]	Corr. Area	% of Total	Average Size [bp]	Size distribution in CV [%]	Conc. [pg/µl]	Molarity [pmol/l]	Color
153	2,141	9,858.5	99	627	52.7	6,882.56	24,256.4	

Peak table for sample 3 : P3

Peak	Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1	35	125.00	5,411.3	Lower Marker
2	59	13.78	354.2	
3	194	198.64	1,550.9	
4	205	76.37	564.0	
5	226	189.26	1,269.4	
6	234	112.06	726.8	
7	256	229.92	1,360.0	
8	287	322.99	1,705.2	
9	303	129.90	649.0	
10	318	197.03	940.2	
11	329	153.57	708.2	
12	341	121.66	539.8	
13	354	309.92	1,327.2	
14	378	131.54	526.7	
15	401	108.63	410.8	
16	411	186.12	686.2	
17	440	244.27	841.2	
18	480	257.10	811.1	
19	500	271.37	822.4	
20	540	270.63	758.6	
21	636	751.51	1,791.1	
22	666	269.19	612.2	
23	810	577.31	1,079.2	
24	933	1,191.00	1,933.8	
25	10,380	75.00	10.9	Upper Marker

Peak	Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
26	12,763	0.00	0.0	

Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE04103941_2013-05-27_02-38-15.xad

Created: 5/27/2013 2:38:15 AM
Modified: 5/27/2013 3:58:57 AM

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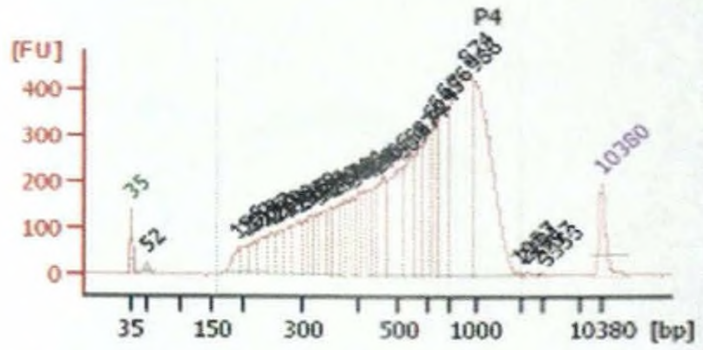

Overall Results for sample 4 : P4

Number of peaks found:	33
Noise:	0.9
Corr. Area 1:	9,915.0

Region table for sample 4 : P4

From [bp]	To [bp]	Corr. Area	% of Total	Average Size [bp]	Size distribution in CV [%]	Conc. [pg/µl]	Molarity [pmol/l]	Color
158	1,998	9,915.0	99	653	50.5	4,923.52	16,419.7	

Peak table for sample 4 : P4

Peak	Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1	35	125.00	5,411.3	Lower Marker
2	52	46.24	1,351.3	
3	195	83.03	645.5	
4	207	78.93	576.6	
5	218	72.26	501.7	
6	237	127.15	811.3	
7	251	116.77	704.0	
8	260	83.55	486.3	
9	278	120.57	657.7	
10	292	127.48	661.0	
11	302	73.27	367.6	
12	313	98.40	476.0	
13	326	88.56	412.0	
14	334	101.73	462.1	
15	349	107.92	468.1	
16	362	122.53	513.1	
17	374	90.03	365.0	
18	388	78.09	304.9	
19	401	118.21	446.8	
20	424	95.94	343.1	
21	440	83.96	289.2	
22	466	210.26	682.9	
23	509	299.65	891.3	
24	547	221.07	612.5	
25	574	176.16	465.4	
26	614	221.11	545.7	
27	645	149.28	350.7	
28	676	272.98	612.0	
29	874	744.37	1,290.7	
30	986	587.95	903.6	
31	1,957	1.61	1.2	
32	2,201	1.64	1.1	
33	2,757	1.46	0.8	
34	3,353	1.63	0.7	
35	10,380	75.00	10.9	Upper Marker

Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE04103941_2013-05-27_02-38-15.xad

Created: 5/27/2013 2:38:15 AM
Modified: 5/27/2013 3:58:57 AM

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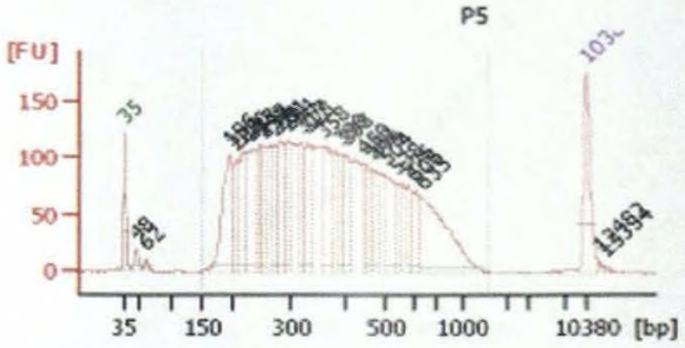


Overall Results for sample 5 : P5

Number of peaks found:	26
Noise:	0.8
Corr. Area 1:	4,468.8

Region table for sample 5 : P5

From [bp]	To [bp]	Corr. Area	% of Total	Average Size [bp]	Size distribution in CV [%]	Conc. [pg/µl]	Molarity [pmol/l]	Color
152	1,605	4,468.8	98	396	44.4	2,907.34	13,968.9	

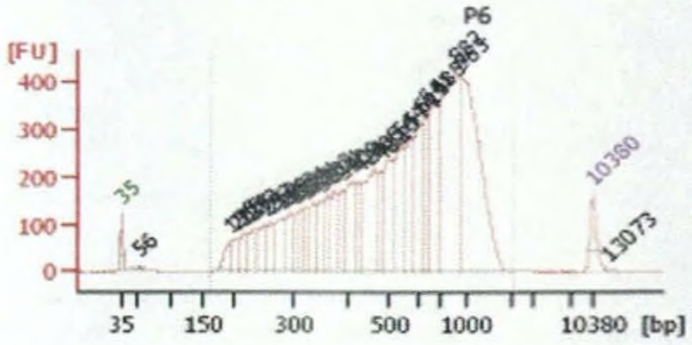
Peak table for sample 5 : P5

Peak	Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1	35	125.00	5,411.3	Lower Marker
2	48	35.16	1,105.2	
3	62	16.70	409.9	
4	196	231.69	1,790.0	
5	208	109.07	796.2	
6	220	126.10	867.2	
7	233	207.14	1,346.5	
8	248	92.54	564.8	
9	253	85.34	510.8	
10	271	88.74	496.6	
11	281	131.78	710.4	
12	294	110.97	572.2	
13	317	97.31	465.8	
14	328	94.00	433.6	
15	358	169.51	718.0	
16	390	62.23	242.0	
17	398	89.65	341.7	
18	450	49.66	167.1	
19	461	51.51	169.4	
20	479	63.15	199.8	
21	504	92.34	277.7	
22	538	54.69	154.0	
23	576	48.53	127.7	
24	606	48.81	122.1	
25	635	188.26	449.0	
26	10,380	75.00	10.9	Upper Marker
27	12,482	0.00	0.0	
28	13,334	0.00	0.0	

Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE04103941_2013-05-27_02-38-15.xad

Created: 5/27/2013 2:38:15 AM
Modified: 5/27/2013 3:58:57 AM

Electropherogram Summary Continued ...



Overall Results for sample 6 : **P6**

Number of peaks found:	30
Noise:	0.5
Corr. Area 1:	9,874.0

Region table for sample 6 : **P6**

From [bp]	To [bp]	Corr. Area	% of Total	Average Size [bp]	Size distribution in CV [%]	Conc. [pg/µl]	Molarity [pmol/l]	Color
165	2,196	9,874.0	99	640	50.4	6,252.40	21,145.5	

Peak table for sample 6 : **P6**

Peak	Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1	35	125.00	5,411.3	Lower Marker
2	56	23.11	626.8	
3	196	112.41	869.5	
4	205	96.47	713.2	
5	218	99.47	690.0	
6	233	117.68	766.8	
7	252	163.04	979.7	
8	262	99.60	576.1	
9	280	179.94	972.5	
10	293	131.32	680.0	
11	302	104.78	525.7	
12	314	130.97	631.9	
13	323	95.42	447.0	
14	334	119.90	543.7	
15	351	171.58	741.0	
16	362	139.72	585.5	
17	374	126.10	511.4	
18	390	118.03	458.9	
19	409	223.95	829.0	
20	424	117.94	421.6	
21	466	399.38	1,297.2	
22	482	117.66	369.9	
23	510	258.10	766.2	
24	544	312.31	870.4	
25	574	188.29	496.6	
26	615	321.14	791.8	
27	641	168.10	397.5	
28	685	361.15	799.4	
29	882	807.98	1,387.4	
30	963	764.11	1,202.6	
31	10,380	75.00	10.9	Upper Marker
32	13,073	0.00	0.0	

Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE04103941_2013-05-27_02-38-15.xad

Created: 5/27/2013 2:38:15 AM
Modified: 5/27/2013 3:58:57 AM

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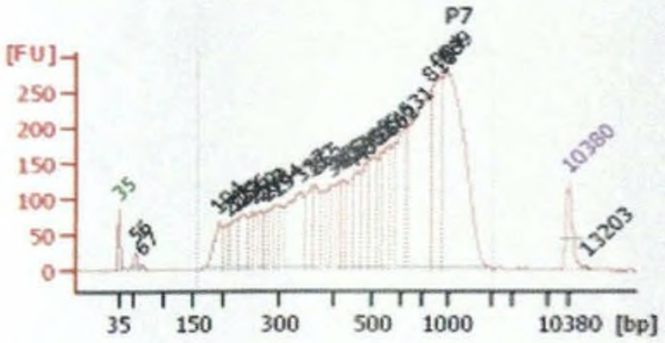

Overall Results for sample 7 : P7

Number of peaks found:	27
Noise:	0.8
Corr. Area 1:	6,923.8

Region table for sample 7 : P7

From [bp]	To [bp]	Corr. Area	% of Total	Average Size [bp]	Size distribution in CV [%]	Conc. [pg/µl]	Molarity [pmol/l]	Color
161	2,120	6,923.8	98	645	54.1	5,747.97	20,241.4	

Peak table for sample 7 : P7

Peak	Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1	35	125.00	5,411.3	Lower Marker
2	56	69.02	1,867.3	
3	67	17.93	406.5	
4	194	176.42	1,374.5	
5	208	105.29	765.2	
6	222	129.90	886.4	
7	236	202.96	1,305.2	
8	252	116.24	698.2	
9	267	99.31	563.5	
10	278	91.24	498.1	
11	294	128.78	663.3	
12	337	375.16	1,684.7	
13	357	172.99	734.3	
14	365	154.74	642.3	
15	393	117.60	453.8	
16	415	114.80	419.5	
17	427	115.61	409.9	
18	447	107.70	365.2	
19	466	158.21	514.1	
20	492	217.99	671.9	
21	515	177.73	522.5	
22	534	127.19	360.9	
23	562	188.03	506.5	
24	631	290.90	698.3	
25	810	655.31	1,225.7	
26	904	372.28	624.3	
27	989	773.62	1,185.7	
28	10,380	75.00	10.9	Upper Marker
29	13,203	0.00	0.0	

Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE04103941_2013-05-27_02-38-15.xad

Created: 5/27/2013 2:38:15 AM
Modified: 5/27/2013 3:58:57 AM

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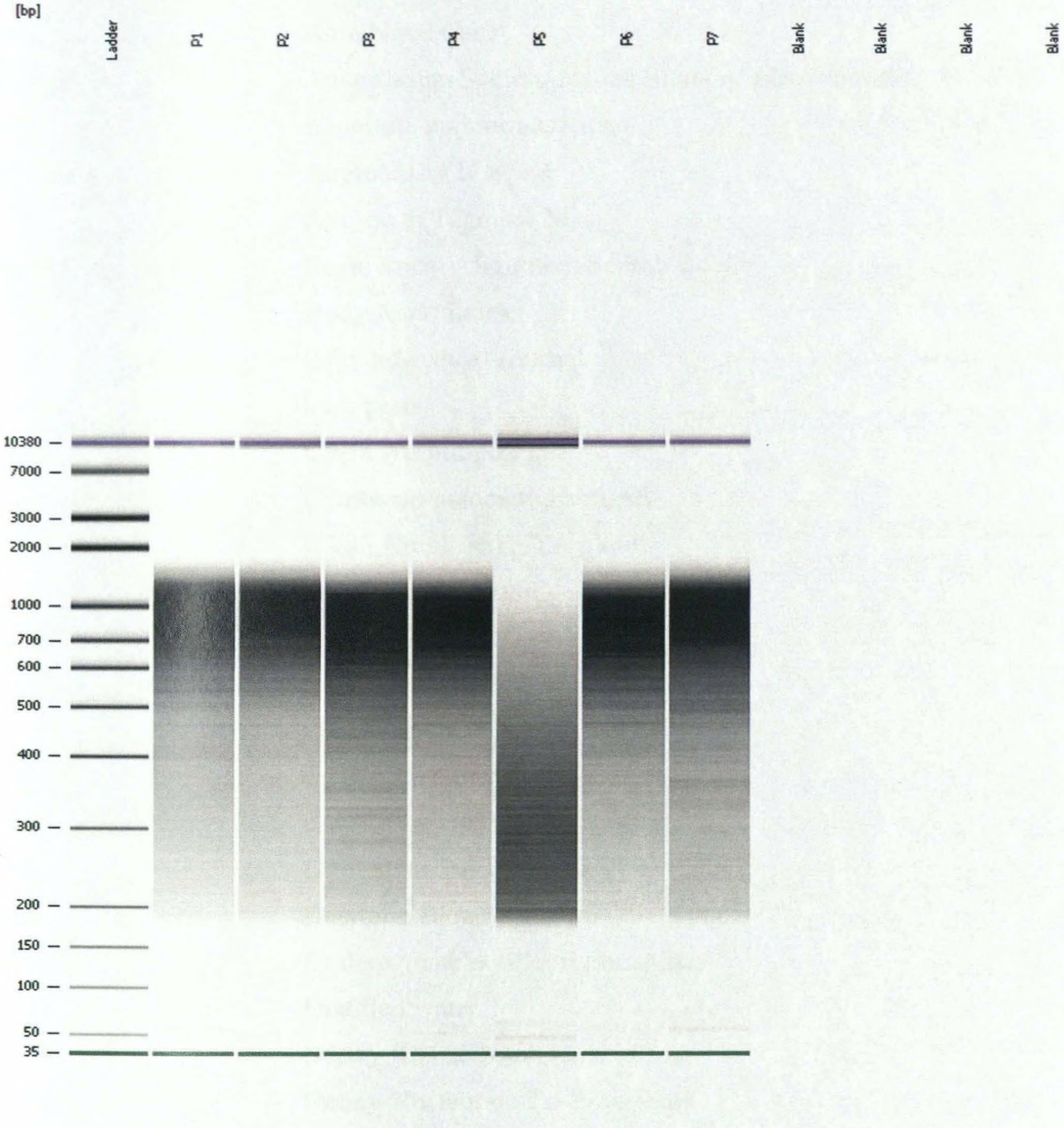


7.3 ABBREVIATIONS

ABI	Applied Bio-Systems
ALT	Alanine aminotransferase
ANC	Ante Natal Care
ASSHP	Australasian Society for the Study of Hypertension
AST	Aspartate aminotransferase
AT1-AAs	Angiotensin II type I
ATM	Amplicon Tagment Mix
BLAST	Basic Local Alignment Search Tool
BMI	Body Mass Index
BMJ	British Medical Journal
bp	base pairs
CAA	Clean Amplified Plate
CagA	Cytotoxin-associated antigen
CAN	Clean Amplified NTA plate
CI	Confidence interval
CKD	Chronic Kidney Disease
CLC	Cell Lysis Buffer
cm	Centi meter
CRP	C- Reactive Protein
CVD	Cardio Vascular Disease
DAL	Diluted Amplicon Library
DBP	Diastolic Blood Pressure
ddNTP	Di deoxy nucleotide tri phosphate
dH_2O	Distilled water
DNA	Deoxy Ribose Nucleic Acid
dNTP	Deoxy Nucleotide Tri Phosphate
E	Eclampsia
ERB	Endo-toxin Removal Wash
Exo 1	Exo-nuclease 1
FHB	Family Health Bureau
FVW	Flow Velocity Waveforms
GH	Gestational Hypertension

(gDNA)	genomic DNA
h	hours
HCT	Heat Coagulation Test
HDL	High density lipoprotein
HELLP	Hemolysis Elevated Liver enzymes Low platelet count
HT1	Hybridization buffer
i	index
ICU	Intensive Care Unit
IL	Inflammatory cytokine interleukin
ISSHP	International Society for the Study of Hypertension in Pregnancy
IUGR	Intra Uterine Growth Reduction
K4	Korotkoff phase 4
kg	Kilogram
km2	Kilometer square
LNA1	Library Normalization Additives 1
LNB1	Library Normalization Beads
LNW1	Library Normalization Wash
LSCS	Lower Segment Caesarean Section
mg	Milligram
MIAC	Microbial invasion of the amniotic cavity
µg	Micro gram
ml	Milliliter
ng	Nano gram
NGS	Next Generation Sequencing
NHBPEP	National High Blood Pressure Education Programme
NICE	National Institute for Health Services and Clinical Excellence
NICU	Neonatal Intensive Care Unit
NPM	Nextera PCR Master Mix
NSC	Neutralization solution
NT	Neutralize Tagment Buffer
NTA	Nextera XT Tagment Amplicon Plate
OR	Odds ratio
P	Probability

P_1C_0	primiparous
PAL	Pooled Amplicon Library
PBU	Perinatal Baby Unit
PE	Pre-eclampsia
PET	Pre-eclamptic toxemia
PGE2	Prostaglandin E2
PGM	Personal Genome Machine
PIH	Pregnancy Induced Hypertension
PLGF	Placental Growth Factor
PMR	Prenatal Mortality Rate
qRT PCR	Real time polymerase chain reaction
RR	Relative risk
rRNA	ribosomal Ribo Nucleic Acid
RUPP	Reduced Uterine Perfusion Pressure
SAP	Shrimp Alkaline Phosphatase
SBP	Systolic Blood Pressure
sEng	Soluble endoglin
sFlt-1	Soluble forms like tyrosine kinase-1
SGA	Small for Gestational Age
SGP	StoraGe Plate
SLE	Systemic Lupus Erythematous
SPE	Superimposed Pre-eclampsia
STNM	Syncytiotrophoblast microparticles
TBE	Tris Borate EDTA buffer
TD	Tagment DNA buffer
TGF	Transforming Growth Factor β
TNF-α	Tumor Necrosis Factor
U/L	Units per liter
V	Variable regions
VEGF	Vascular Endothelial Growth Factor
VLDL	Very low density lipoprotein
WHO	World Health Organization
$	Dollar

µg	micro gram
≥	More than or equal to
%	Percentage

7.4 BUFFERS AND CULTURE MEDIA

Luria-Bertani (LB) Medium

Bacto-tryptone	10	g
Bacto-yeast extract	5	g
Sodium chloride	10	g

Adjust the pH to 7 .0 with 5N sodium hydroxide

SOC Medium (100ml)

Bacto-tryptone	2	g
Bacto-yeast extract	0.5	g
Sodium chloride	1	ml
1M potassium chloride	1	ml
2M $MgCl_2$	1	ml
2M Glucose	20	ml
dH_2O	97	ml

Adjust the pH to 7 .0 with 5N sodium hydroxide

IPTG Stock solution (0.1M)

IPTG	1.2	g
dH_2O	50	ml

Filter sterilize and store at 4°C

X-Gal (2ml)

5-bromo-4-chloro-3-indoyl-β-D-galactoside	100	mg
N,N'-dimethyl formsmide	2	ml

Dissolve and cover with aluminium foil and store at 20°C

LB Broth with Ampicillin

LB broth	100	ml
Ampicillin	100	µl/ml

mixed 100µl/ml ampicillin in autoclaved cooled LB broth

LB plates with Ampicillin

LB broth prepared	100	ml
Lactose agar	1.5	g
Ampicillin	100	µl/ml

Autoclave LB broth mixed with agar. Allow to cool and add ampicillin before poring the plates

LB plates with Aampicillin/IPTG/X-Gal

IPTG	100	µl
X-Gal	20	µl

Spread IPTG/X-Gal on the LB/Amp plates prepared

Im Amp Blue plates- Invitrogen

Im Amp Blue media	1	Packet
dH_2O (Autoclaved)	200	ml

Heat in a microwave for 2 minutes and pour on to plates

10X TBE buffer (1L)

Tris base	108	g
Boric acid	55	g
0.5M EDTA	40	ml
dH_2O	960	ml

Mix using a magnetic stirrer and autoclave for 20 minutes

7.5 LIST OF SUPPLIERS

Applied Biosystems	7 Kingsland Grange, Woolston, Warrington, Cheshire, WA1 7SR, UK
GE Healthcare in Uk	Pollards Wood. Nightingales Lane, Chalfont St, Giles, Buckinghamshire, HP8 4SP
Illumina Singapore Ltd	11 Biopolis Way, #09-05 Helios, Singapore 138667
Invitrogen Ltd.	3 Fountain Drive, Inchinnan Business Park, Paisley, PA4 9RF, UK
Molzym GmbH & Co. KG	Mary-Astell-Straße 10, 28359 Bremen, Germany
Promega UK Ltd	Delta House, Chilworth Research Centre, Southampton SO16 7NS, UK.
Qiagen	QIAGEN House, Fleming Way, Crawley, West Sussex, RH10 9NQ, UK.

7.6 PATIENT INFORMATION SHEETS, CONSENT FORM & DATA COLLECTION SHEETS

7.6.1 Information sheet and consent form (Sinhala)

<u>විස්තර පත්‍රිකාව</u>

ගර්භ විෂ රෝගයට ගොදුරු වූ ගර්භණී ස්ත්‍රීන් සහ නිරෝගී ගර්භණී ස්ත්‍රීන්ගේ රුධිරය මුත්‍රා කලළාවාරික තරලයල වැදෑමහේ පිවත් වන ක්ෂුද්‍ර ජීවීන්ගේන් ගර්භවිෂ රෝගයට සිදුවන බලපෑම පිළිබඳ සංසංදනාත්මක ප්‍රවේණි විද්‍යාත්මක පරීක්ෂණය

රන්මලි අමරසේකර වන මම කොළඹ වෛද්‍ය පීඨයේ පශ්චාත් උපාධි අපේක්ෂිකාවක් වන අතර මහාචාර්ය රොහාන් ජයසේකර වෛද්‍ය වජිර දිසානායක මහාචාර්ය හේමන්ත සෙනානායක සහ මා විසින් සිදු කරනු ලබන ඉහත සඳහන් පරීක්සණයට සහභාගි වන ලෙෂ ඔබට ආරාධනා කරමි

1 පර්යේෂණයේ අරමුණ

මෙම පර්යේෂණයේ අරමුණ වන්නේ ගර්භ විෂ රෝගය සහ ක්ෂුද්‍ර ජීවී ආසාදිත රෝග අතර ඇති සම්බන්ධතාවය පරීක්ෂා කිරීමයිග

2 ස්වේච්ඡා සහභාගීත්වය

මෙම පර්යේෂණය සඳහා ඔබගේ සහභාගීත්වය සිදුවිය යුත්තේ ස්වේච්ඡාවෙනිග ඔබ අකැමැති නම් සහභාගී නොවී සිටිය හැකි අතර කැමැත්තෙන් සහභාගී වුවත් අවශ්‍ය නම් අතරමගදීල අවශ්‍ය වේලාවකදී ඉවත්වීමේ අයිතිය ද ඔබ සතුයිග සහභාගී නොවීම නිසා ඔබට ලැබිය යුතු වෛද්‍ය ප්‍රතිකාර හා සේවාවන්හි කිසිදු වෙනසක් සිදු නොවනු ඇතග ඔබ ඉවත් වීමට තිරණය කල විට කරුණාකර හැකි ඉක්මනින් ඒ බව අපට දන්වන්නග

3 පර්යේෂණ කාලය ක්‍රියාවලිය හා සහභාගීවන්නන්ගේ වගකීම්

ඔබගේ වෛද්‍ය වාර්තා සහ සායනික විස්තර සටහන් ගැනීමට රුධිර සාම්පලයක් මුත්‍රා සාම්පලයක් කලළාවාරික තරලයල සහ වැදෑමහේ සාම්පලයක් ලබා ගැනීමට අනුමැතිය අපට අවශ්‍ය වේග වෙනත් කිසි දෙයක් අප ඔබෙන් බලාපොරොත්තු නොවේ

4 ඔබට ලැබිය හැකි වාසි

ඔබ මෙම පර්යේෂණයට සහභාගීවීමෙන් ගර්භ විෂ රෝගයට සම්බන්ධ ක්ෂුද්‍ර ජීවී හඳුනා ගැනීමට හැකි වන අතර එම දැනුම උපයෝගිකොටගෙන ඔබට සහ ඔබගේ වෛද්‍යවරයාට එවැනි අවස්ථා වලකාලීමට උපකාර විය හැක.

5 අවාසි හා අපහසුතා

ඔබටවත් ඔබගේ බිළිදාටවත් කිසිදු විදියක අවාසියක් අපහසුතාවයක් හෝ අතුරු ආබාධයක් මෙම පරීක්ෂණයට හාජනය වීමෙන් සිදු නොවේග මෙම පරයේෂණයට සහභාගීවීම වෙනුවෙන් ඔබට කිසිදු ගෙවීමක් සිදු නොකෙරේග

6 රහස්‍යයභාවය

ඔබේ සියලුම වාර්තාවල රහස්‍යයභාවය සුරක්ෂිතීමට අප බැදී සිටිමුග ඔබ සපයන තොරතුරු වලින් ඔබව හඳුනා ගත නොහැකි වන අතර ඔබ හඳුනා ගත හැකි තොරතුරු වේනම් පිටස්තර කිසිවෙකුට ලබා ගැනීමට නොහැකි පරිදි ආරක්ෂා කර තබා ගන්නෙමුග මෙම තොරතුරු කිසිවිටක ඔබව හඳුනා ගත පරිදි ඔබගේ අනුමැතිය නොමැතිව කිසිම ආකාරයකින් පල නොකරන්නෙමුග

8 පරයේෂණයෙන් ඉවත් වීම

දැන් කැමැත්ත දුන්නත් පසුව අකමැතියැයි සිතුනොත්, අපට දන්වා පරයේෂණයෙන් ඉවත් විය හැක.මේ නිසා ඔබට ලැබිය යුතු ප්‍රතිකාර හෝ සේවාවල කිසිදු වෙනසක් සිදු නොවනු ඇතග ඔබ ඉවත් වීමට තිරණය කල විට කරුණාකර හැකි ඉක්මනින් ඒ බව අපට දන්වන්නග

9 පැහැදිලි කිරීම්

ඔබට අදාළ පරයේෂණය පිළිබඳව ප්‍රශ්න ඇත්නම් හෝ වැඩි විස්තර දැන ගැනීමට අවශ්‍ය නම් හෝ පරයේෂණයෙන් ඉවත් වීමට අවශ්‍ය නම් **0112689545** ඔස්සේ මා අමතීමට පසුබට නොවන්න

අනුමැති පත්‍රිකාව

ගර්භ විෂ රෝගය සහ ක්ෂුද්‍ර ජීවී ආසාදිත රෝග අතර ඇති සම්බන්ධතාවය පරීක්ෂා කිරීමට මෙම පත්‍රිකාව පර්යේෂණයට සහභාගී වන පුද්ගලයා විසින් පිරවිය යුතුයිග (අදාළ පිළිතුර රවුම් කරන්නග)

1. ඔබ විස්තර පත්‍රිකාව කියෙව්වාදෑ ඔව් \ නැහැ
   (කරුණාකර එහි පිටපතක් ඔබ ළඟ තබා ගන්නග)

2. මෙම පර්යේෂණය පිළිබඳව සාකච්ඡා කිරීමට හා ඔබට ඇති ප්‍රශ්න ඇසීමට ඔබට අවස්ථාව ලැබුනේද රෑඔව් \ නැහැ

3. ඔබට තිබූ ප්‍රශ්න සියල්ලටම සෑහීමකට පත් විය හැකි පිළිතුරු ලැබුනේදරෑඔව් \ නැහැ

4. පර්යේෂණය පිළිබඳව ඔබට අවශ්‍ය තරමට තොරතුරු ලැබුනේද රෑ ඔව් \ නැහැ

5. ඔබට අවශ්‍ය අවස්ථාවකදී හේතු ඉදිරිපත් කිරීමකින් තොරව හා ඔබට ලැබිය යුතු වෛද්‍ය ප්‍රතිකාර වලට බාධා වීමකින් තොරව ඔබට මෙම පර්යෙෂණයෙන් ඉවත්විය හැකි බව ඔබ තේරුම් ගත්තාදෑ ඔව් \ නැහැ

6. මෙම පර්යේෂණයට ඔබගේ සහභාගීත්වය පිළිබඳ තොරතුරු හා ඔබගේ අනෙකුත් වෛද්‍ය වාර්තාවල කොටස් වෙනත් පර්යේෂණ සහායකයින්ට පිරික්සා බැලීමට සිදුවිය හැක එහෙත් ඔබගේ සියලුම පෞද්ගලික තොරතුරුවල රහස්‍යභාවය සුරැකෙනු ඇතග ඔබ ඉහත සඳහන් අයට ඔබගේ වාර්තා පිරික්සීමට අනුමැතිය දීමට කැමතිදෑ ඔව් \ නැහැ

7. ඔබට පර්යේෂණයට සහභාගි වීම \ නොවීම තීරණය කිරීමට අවශ්‍ය තරම් කාලය ලැබුනේද රෑඔව් \ නැහැ
8. ඔබ මෙම පර්යේෂණයට සහභාගී වීමට කැමතිද රෑ ඔව් \ නැහැ

සහභාගීවන්නගේ අත්සන..... දිනය .....

සම්පූර්ණ නම.....

පර්යේෂණයට මා විසින් මෙම පර්යේෂණය පිළිබඳවදව මෙම ස්වේච්ඡා සහභාගීවන්නා හට කරුණු පැහැදිලි කරනු ලැබූ අතර ඇය මෙම පර්යෙෂණයට සහභාගී වීමට එකඟ වූවාය

පර්යේෂකගේ අත්සන .....දිනය .....

සම්පූර්ණ නම .....

7.6.2 Information sheet and consent form (English)

Information Sheet/Consent Form

A comparative study of viruses, bacteria and fungi present in blood, urine, amniotic fluid, and placental tissue of women with pre-eclampsia and normal pregnant women using molecular genetic techniques

I am Ms. Ranmalee Amarasekara, attached to the Human Genetics Unit, Faculty of Medicine University of Colombo reading for my PhD. I would like to invite you to take part in the research study titled "A comparative study of viruses, bacteria and fungi present in blood, urine, amniotic fluid, and placental tissue of women with pre-eclampsia and normal pregnant women using molecular genetic techniques" conducted by Prof. Rohan W Jayasekara, Dr. Vajira H. W. Dissanayake, Prof. Hemantha Senanayake and myself.

1. Purpose of the study

The purpose of this research is to find out whether pre-eclampia is caused by an infective organism.

2. Voluntary participation

Your participation in this study is voluntary. You are free not to participate at all or to withdraw from the study at any time despite consenting to take part earlier. There will be no loss of medical care or any other available treatment for your illness or condition to which you are otherwise entitled. If you decide not to participate or withdraw from the study you may do so at any time.

3. Duration, procedures of the study and participant's responsibilities

If you agree to participate then we will do the following at the time of delivering your baby by caesarian section:

- When a canula is inserted to one of your veins to give you saline we will take 3ml of blood.
- When the catheter is inserted to your bladder to remove urine we will take 10 ml of urine.
- When the amniotic cavity is opened to deliver the baby we will take 10 ml of amniotic fluid.
- When the placenta is taken out we will take the entire placenta.

We will be testing these to find out whether there are any infectious organisms present in them. In addition to these we will record clinical information relating to you.

4. Potential benefits

There are no direct benefits to you from participation in this study. However, your participation in this study may contribute to understanding what causes pre-eclampsia

thereby benefiting you (if you plan to have more children) and/or other pregnant women in the future.

5. Risks, hazards and discomforts

There are no additional risks, hazards and discomforts for your or your baby as a result of participating in this study.

6. Confidentiality

The information we gather will never be used in such a way that your identity and information would be released to a third party.

7. Withdrawal from the study

Although you agree to participate now you are free to change your mind later and withdraw from the study. All you have to do is call one of us and let us know that you want to withdraw.

8. Clarification

If you have questions about any of the tests / procedures or information, please feel free to ask one of us by calling 011 2689 545

9. To be complete

a. By the participant

The participant should complete the whole of this sheet himself/herself.

1. Have you read the information sheet? (Please keep a copy for yourself) YES/NO

2. Have you had an opportunity to discuss this study and ask any questions? YES/NO

3. Have you had satisfactory answers to all your questions? YES/NO

4. Have you received enough information about the study? YES/NO

5. Who explained the study to you? .....

6. Do you understand that you are free to withdraw from the study at any time without having to give a reason and without affecting your future medical care? YES/NO

7. Sections of your medical notes, including those held by the investigators relating to your participation in this study may be examined by other research assistants. All personal details will be treated as STRICTLY CONFIDENTIAL. Do you give your permission for these individuals to have access to your records? YES/NO

8. Have you had sufficient time to come to your decision? YES/NO

9. Do you agree to take part in this study?YES/NO

Participant's signature……..……Date……

Name (BLOCK CAPITALS)……

b. By the investigator

I have explained the study to the above volunteer and she has indicated her willingness to take part.

Signature of investigator……...……..Date……

Name (BLOCK CAPITALS)……

7.6.3 Information sheet and consent form (Tamil)

அறிவுறுத்தல் படிவம் / சம்மதப் படிவம்

"நுண்ணணுத் தொடர்பான பரம்பரையலகு வழிமுறைகளை பயன்படுத்தி, சாதாரண கர்ப்பிணிப் பெண்ணில் பக்ரீறியா, பங்கசு, வைரசுத் தொற்று வாய்ப்புள்ளதா என்பதை அறிவதற்காக, அப்பெண்ணின் குருதி, சிறுநீர், கருப்பையில் கருவைச் சுற்றியுள்ள திரவம், தொப்புள்கொடி ஆகியவற்றை பாவித்து ஒரு ஒப்பிடும் தன்மையுள்ள ஆய்வு" - இவ்வகைத் தொற்று Pre eclampsia என அழைக்கப்படும்.

நான் செல்வி றண்மலி அமரசேகர – மனித மரபியல் அலகு, மருத்துவ பீடம், கொழும்பு பல்கலைக்கழகத்தில் உயர் பட்டப் படிப்புக்காக இந்த ஆய்வை மேற்கொள்கிறேன். நான் மேற்குறிப்பிட்ட தலைப்புத் தொடர்பாக, உங்களைப் பங்குபற்றுவதற்காக அன்புடன் அழைக்கிறேன். இந்த ஆய்வு பேராசிரியர். றொகான் டபிள்யூ. ஜயசேகர, வைத்தியர் வஜிர எச். டபிள்யூ. திசனாயக்க, பேராசிரியர். ஹேமந்த சேனநாயக்க மற்றும் என்னால் வழிநடத்தப்படுகிறது.

1 ஆய்வின் நோக்கம் :-

Pre cclampsia – இது தொற்றுள்ள நுண்ணங்கிகளினாலோ ஏற்படுகிறது என்பதை அறிவதற்காக மேற்கொள்ளப்படுகிறது.

2 சுயவிருப்பப்படி பங்குபற்றல் :-

இந்தக் கற்கையில் உங்கள் பங்குபற்றல் தன்னிச்சையானது. நீங்கள் சுயேச்சையாக இந்த ஆய்வில் இருந்து விலக முற்பட்டால் விரைவாக விலகிக் கொள்ளமுடியும். உங்களது வலிக்கோ அல்லது எந்தவொரு சந்தர்ப்பத்திலோ நீங்கள் பெயர் கொடுக்காவிடினும், மருத்துவக் கவனிப்பில் இழப்போ அல்லது சிகிச்சை முறையில் இழப்போ ஏற்படமாட்டாது. நீங்கள் பங்குபற்றவில்லை என தீர்மானித்து, கற்கையிலிருந்து விலக முற்பட்டால், அதை நீங்கள் எந்த நேரத்திலும் மேற்கொள்ளலாம்.

3 நீடிக்கும் காலம், படிமுறைகள், பங்குபற்றாளரின் கடமைகள் :-

நீங்கள் இந்த ஆய்வில் பங்குபற்றுவதாக இருந்தால் அறுவைச் சிகிச்சை குழுவினரால் நீங்கள் குழந்தையை பிரசவிக்கும் நேரத்தில் பின்வரும் படிமுறைகள் மேற்கொள்ளப்படும்.

- உட்புகுத்தியைப் பயன்படுத்தி இரத்தக்குழாய் ஒன்றினுள் சேலைன் ஏற்றப்படும். பின்னர், நாங்கள் 3 மில்லி லீற்றர் குருதியை உங்களிடமிருந்து சேகரிப்போம்.
- உட்புகுத்தப்படும் வடிகுழாய் ஒன்றின் மூலம் சிறுநீர்ப்பையிலிருந்து 10 மில்லி லீற்றர் சிறுநீர் எடுக்கப்படும்.
- பிரசவத்திற்காக கருவைச் சுற்றியுள்ள திரவத்தில் 10 மில்லி லீற்றர் சேகரிக்கப்படும்.
- தொப்புள்கொடி வெளியே வரும்போது, எங்களால் முழுமையான தொப்புள்கொடியை பெற்றுக்கொள்ள முடியும்.

பின்னர், இவை அனைத்தும் பரிசோதிக்கப்படும். இவற்றில் இருந்து அந்தப் பெண்ணிற்கு தொற்றுள்ள நுண்ணங்கியின் பாதிப்பு ஏதாவது இருக்கின்றதா என்பதை அறிந்து

கொள்ளமுடியும். அத்துடன், சிகிச்சைமுறை தொடர்பான தகவல்கள் உங்களுக்கு சார்பாக பதியப்படும்.

4 சாத்தியமான அனுகூலங்கள்:-

உங்களுக்கு நேரடியான நன்மைகள் இந்த ஆய்வில் இல்லை. ஆயினும் உங்கள் பங்குபற்றல் ஒரு விழிப்புணர்வை ஏற்படுத்தும். Pre cclampsia பற்றி அறிவதற்கு இது ஒரு நல்ல சந்தர்ப்பமாக இருக்கும் (உங்களுக்கு நிறைய பிள்ளைகள் பெறவேண்டும் என்ற எண்ணம் இருந்தால்) அல்லது எதிர்காலத்தில் மற்றைய கர்ப்பிணித் தாய்மாருக்கு ஒரு விழிப்புணர்வை ஏற்படுத்தும் ஆய்வாக அமைய முடியும்.

5 அபாயம், ஆபத்து, அசௌகரியங்கள்:-

இந்த ஆய்வில் பங்குபற்றுவதால், உங்களுக்கோ அல்லது உங்கள் குழந்தைக்கோ எந்தவொரு அபாயமோ, ஆபத்தோ, அசௌகரியமோ ஏற்படமாட்டாது.

6 இரகசியம் பேணல்:-

உங்களை அடையாளப்படுத்தும் எந்தவொரு தகவலும் ஒருபோதும் வேறொருவருக்கு வெளியிடப்படமாட்டாது.

7 ஆய்விலிருந்து விலகுதல்:-

முதலில் இந்த ஆய்வில் பங்குபற்றுவதாக இருந்து, பின்னர் இதில் பங்

குபற்ற இயலாது என உங்கள் மனதில் தோன்றினால் நீங்கள் எங்களைத் தொடர்பு கொண்டு உங்கள் விருப்பத்தை தயங்காமல் தெரிவித்துக் கொள்ளமுடியும்.

8 சந்தேகங்கள்:-

இந்த ஆயவிலோ அல்லது படிமுறைகளிலோ தகவல்களிலோ ஏதாவது சந்தேகங்கள் இருந்தால், தயவு செய்து இந்த தொடர்பு இலக்கத்திற்கு தொடர்பு கொண்டு அறிந்து கொள்ள முடியும்.

9 பூர்த்தி செய்ய வேண்டுயவை:-

அ) பங்குபற்றாளரினால்,

பங்குபற்றாளரினால், கட்டாயமாக கீழ்க் கொடுக்கப்பட்ட கேள்விகள் அனைத்தும் தாமாகவே பூர்த்தி செய்யப்படல் வேண்டும்.

1 நீங்கள் மேற்தரப்பட்ட தகவல்கள் அனைத்தையும் வாசித்தீர்களா?
ஆம் / இல்லை

(தயவு செய்து இதன் பிரதி ஒன்றை உங்களுடன் வைத்திருக்கவும்)

2 இந்த ஆய்வைப் பற்றி கலந்தாலோசிப்பதற்கு அல்லது அது தொடர்பாக ஏதாவது கேள்வி கேட்பதற்கு நீங்கள் ஏதாவது வாய்ப்பு வைத்திருக்கிறீர்களா?
ஆம் / இல்லை

3 கேள்விகள் அனைத்திற்கும் திருப்தியான பதில்கள் உங்களிடம் இருக்கின்றதா?
ஆம் / இல்லை

4 நீங்கள் இந்த ஆய்வு தொடர்பாக தேவையான அளவு தகவல்களைப் பெற்றிருக்கின்றீர்களா?
ஆம் / இல்லை

5 இந்த ஆய்வு பற்றி யார் உங்களுக்கு விளங்கப்படுத்தினார்கள்?

6 எதிர்காலத்தில் உங்களது மருத்துவ பாதுகாப்பை பாதிக்காத விதத்தில், எந்தவொரு காரணங்களையும் காட்டாமல் இந்த ஆய்விலிருந்து விலகிக் கொள்ளலாம் என்பதை நீங்கள் சரியாக விளங்கி கொண்டிருக்கிறீர்களா? ஆம் / இல்லை

7 உங்களது மருத்துவக் குறிப்பு உள்ளடங்கியவை இந்த ஆய்வில் ஆய்வாளரினால் மேற்கொள்ளப்பட்டு, மற்றொரு ஆய்வாளரினாலும் ஆய்வு மேற்கொள்ளப்படலாம். உங்களது தனிப்பட்ட விடயங்கள் யாவும் கண்டிப்பாக இரகசியமான முறையில் பேணப்படும். அதனால் இந்த ஆய்வை மேற்கொள்ள உங்களால் அனுமதி தரமுடியுமா?
ஆம் / இல்லை

8 உங்கள் முடிவை அடைவதற்கு, நீங்கள் தேவையான நேரத்தை செலவழித்தீர்களா?
ஆம் / இல்லை

9 நீங்கள் இந்த ஆய்வில் பங்குபற்ற சம்மதமா?
ஆம் / இல்லை

பங்குபற்றாளரின் கையொப்பம்: ..... திகதி: ....பெயர்:

ஆ) ஆய்வாளரினால்:நான் இந்த ஆய்வு பற்றி இவரிற்கு விளங்கப்படுத்தியுள்ளேன். அவர் தான் பங்குபற்றுவதற்கு தனது விருப்பத்தை தெரிவித்துள்ளார்,

ஆய்வாளரின் கையொப்பம்: ..... திகதி: ..... பெயர்:

7.6.4 Data collection form- Preeclampsia woman

Subject study number……

A comparative study of viruses, bacteria and fungi present in blood, urine, amniotic fluid, and placental tissue of women with pre-eclampsia and normal pregnant women

PHENOTYPIC DATA
Pre-Eclamptic Woman

Screening Number | | | | | / | | | |

Name ……

Date of birth ……/……/……...

Age ……yrs

Parity ……

Address ……

……

Telephone number ……

Hospital number ……

ANC Clinic number ……

PHC Clinic number …… Name ……

Data Protection and Confidentiality After completion of this page, ensure that the subject study number is entered on all pages of this booklet. Then detach this page and store separately from the remainder of the booklet.

Screening Number | | | | | / | | | |

Date of entry to study .....
Date on consent form

INCLUSION CRITERIA

No hypertension of ≥ 140/90 mmHg on Two occasions within 6h apart | |

No proteinuria of ≥ 1in urine-protein Heat coagulation test | |

EXCLUSION CRITERIA

Known risk factors	
(renal disease, chronic disease, persistent proteinuria)	
Essential hypertension requiring medication	
Deep vein thrombosis/pulmonary embolism	
Ischemic heart disease	
Cerebrovascular accidents	
Insulin/non insulin dependent diabetes mellitus	
Diabetes and gestational diabetes	
Body mass index ≥ 30 kg/m^2	
Multiple gestations	
Long term steroid use	
Endocrine disorders	
Valvular heart disease	
Multiple vaginal examinations	
Premature rupture of membranes	

Past medical & surgical history.....

Screening Number [ ][ ][ ][ ] / [ ][ ][ ]

ANTE-NATAL BOOKING

Date of booking [ ][ ]-[ ][ ]-[ ][ ][ ][ ]

Maternal weight in kilograms [ ][ ][ ].[ ] maternal height in meters [ ].[ ][ ]

Body Mass Index (Weight in Kg/Height in Meters^2) [ ][ ]

Dates:

LMP [ ][ ]-[ ][ ]-[ ][ ][ ][ ]

Gestation by dates weeks [ ][ ] days [ ]

SCAN

Date of first scan [ ][ ]-[ ][ ]-[ ][ ][ ][ ]

POA by the date of first scan .....

EDD by scan .....weeks .....days

Gestation by scan .....weeks.....days

Blood pressure at booking:

Systolic [ ][ ][ ] mm Hg

Diastolic [ ][ ][ ] mm Hg

Urinary protein

[ ] 0. Negative or trace

[ ] 1. Not performed or result unknown

[ ] 2. ≥ (+)

Screening Number | | | | | / | | | |

DIAGNOSIS OF PRE-ECLAMPSIA

Date of diagnosis | | | - | | | - | | | | |

Earliest date on which BP and proteinuria fulfill inclusion criteria. Date at either start or finish of 24 hour urine collection is acceptable.

☐ 0. Antenatal ☐ 1. Intrapartum ☐ 2.Postnatal

Gestation at diagnosis weeks days
Calculated from booking scan
Where available | | | |

Blood pressure at diagnosis (not on antihypertensive medication)

BP preferably not on antihypertensive medication, but this is not mandatory. For women who have commenced antihypertensive treatment prior to crossing threshold for proteinuria, see study guidelines.

1. Date | | | - | | | - | | | | |

Time | | | . | |

Systolic | | | mm Hg

Diastolic | | | mm Hg

First and second reading should be taken within 24 hours, and preferably 6 hours apart. Lesser intervals are acceptable where no other readings are available e.g. fulminating pre-eclampsia

2. Date | | | - | | | - | | | | |

Time | | | . | |

Systolic | | | mm Hg

Diastolic | | | mm Hg

Screening Number ☐☐☐☐ / ☐☐☐

Proteinuria at diagnosis

Enter ONE measure of proteinuria only: g/L in a 24 hour collection, or g/24 hours preferred.

Urinary protein in g/L ☐☐.☐☐☐

24 hour collection No / Yes

Heat coagulation protein

☐ 0. Negative or trace

☐ 1. +

☐ 2. ++

☐ 3. +++

☐ 4. ++++

SEVERITY OF DISEASE

Blood pressure at time of highest recorded diastolic blood pressure

Highest BP at or since diagnosis

Date ☐☐-☐☐-☐☐☐☐

☐ 0. Antenatal ☐ 1. Intrapartum ☐ 2.Postnatal

Systolic ☐☐☐ mm Hg

Diastolic ☐☐☐ mm Hg

Highest recorded proteinuria within ± 1 week of delivery

Date ☐☐-☐☐-☐☐☐☐

Screening Number [ ][ ][ ][ ] / [ ][ ][ ]

Heat coagulation protein

0. Negative or trace

[ ] 1. +

[ ] 2. ++

[ ] 3. +++

[ ] 4. ++++

Biochemical data within ± 1 week of delivery

Highest recorded:

			Date
AST	[ ][ ][ ][ ]	U/L	[ ][ ]-[ ][ ]-[ ][ ][ ][ ]
ALT	[ ][ ][ ][ ]	U/L	[ ][ ]-[ ][ ]-[ ][ ][ ][ ]
Urate	[ ][ ][ ][ ]	mmol/L	[ ][ ]-[ ][ ]-[ ][ ][ ][ ]

To convert mmol/L to µmol/L, multiply by 1000 (move the decimal point 3 places to the right)

Creatinine	[ ][ ][ ][ ]	mmol/L	[ ][ ]-[ ][ ]-[ ][ ][ ][ ]
Urea	[ ][ ].[ ]	mmol/L	[ ][ ]-[ ][ ]-[ ][ ][ ][ ]

Lowest recorded:

Platelets	[ ][ ][ ]	x 10^9/L	[ ][ ]-[ ][ ]-[ ][ ][ ][ ]

Admitted to AICU

[ ] 0. No

[ ] 1. Yes

Screening Number [ ][ ][ ][ ] / [ ][ ][ ]

Required renal dialysis
[ ] 0. No
[ ] 1. Yes

PREGNANCY OUTCOME

Date of delivery [ ][ ]-[ ][ ]-[ ][ ][ ][ ]

Gestation at delivery [ ][ ] weeks [ ] days

Calculated from gestation at diagnosis

Reason for caesarean section [ ]

Complications:
[ ] 1. Abruption
[ ] 2. Antenatal eclamptic convulsion
[ ] 3. Intrapartum eclamptic convulsion
[ ] 4. Postpartum eclamptic convulsion

SAMPLING RECORD- for DNA extraction

Venous blood ☐

Amniotic fluid ☐

Urinary sample ☐

Placental tissue ☐

COMMENTS

Record reasons for missing data and any additional relevant comments, including any intervention trials in which the subject participated during this study. ENSURE THAT ANONYMITY IS PRESERVED.

The booklet should be signed when ALL available data have been entered and cross checked with relevant data recorded elsewhere in this booklet and in the phenotyping booklets for the mother of the affected woman and the index affected baby

Signed…….. Date…….

Investigator/ research assistant

7.6.5 Data collection form- Normotensive woman

Subject study number.....

A comparative study of viruses, bacteria and fungi present in blood, urine, amniotic fluid, and placental tissue of women with pre-eclampsia and normal pregnant women

PHENOTYPIC DATA
Normotensive Woman

Screening Number | | | | | / | | |

Name .....

Date of birth ...../...../.....

Age .....yrs

Parity .....

Address .....

.....

.....

Telephone number .....

Hospital number .....

ANC Clinic number .....

PHC Clinic number ..... Name .....

Data Protection and Confidentiality After completion of this page, ensure that the subject study number is entered on all pages of this booklet. Then detach this page and store separately from the remainder of the booklet.

Screening Number | | | | | / | | | |

Date of entry to study .....
Date on consent form

Inclusion Criteria

No hypertension of ≥ 140/90 mmHg on Two occasions within 6h apart | |

No proteinuria of ≥ 1 in urine-protein Heat coagulation test | |

Exclusion criteria

Known risk factors
(renal disease, chronic disease, persistent proteinuria)
Essential hypertension requiring medication
Deep vein thrombosis/pulmonary embolism
Ischemic heart disease
Cerebrovascular accidents
Insulin/non insulin dependent diabetes mellitus
Diabetes and gestational diabetes
Body mass index ≥ 30 kg/m^2
Multiple gestations
Long term steroid use
Endocrine disorders
Valvular heart disease
Multiple vaginal examinations
Premature rupture of membranes

Past medical & surgical history.....

Screening Number [ ][ ][ ][ ] / [ ][ ][ ]

ANTE-NATAL BOOKING

Date of booking [ ][ ]-[ ][ ]-[ ][ ][ ][ ]

Maternal weight **in kilograms** [ ][ ][ ].[ ]

Maternal height **in metres** [ ].[ ][ ]

Body Mass Index (Weight in Kg/Height in Meters^2) [ ][ ]

Dates:

LMP [ ][ ]-[ ][ ]-[ ][ ][ ][ ]

Gestation by dates [ ][ ] **weeks** [ ] **days**

SCAN

Date of first scan [ ][ ]-[ ][ ]-[ ][ ][ ][ ]

POA by the date of first scan .....

EDD by scan .....weeks .....days

Gestation by scan .....weeks.....days

Blood pressure at booking:

Systolic [ ][ ][ ] mm Hg

Diastolic [ ][ ][ ] mm Hg

Urinary protein
- [ ] 0. Negative or trace
- [ ] 1. Not performed or result unknown
- [ ] 2. ≥ (+)

MSU culture
- [ ] 0. Negative/not performed/results unknown
- [ ] 1. Urinary tract infection

Screening Number [ ][ ][ ][ ] / [ ][ ][ ]

PREGNANCY OUTCOME

Date of delivery [ ][ ] - [ ][ ] - [ ][ ][ ][ ]

Gestation at delivery [ ][ ] weeks [ ] days

Calculated from gestation at diagnosis

Reason for caesarean section [ ]

SAMPLING RECORD- for DNA extraction

Venous blood [ ]

Amniotic fluid [ ]

Urinary sample [ ]

Placental tissue [ ]

COMMENTS

Record reasons for missing data and any additional relevant comments, including any intervention trials in which the subject participated during this study. ENSURE THAT ANONYMITY IS PRESERVED.

The booklet should be signed when ALL available data have been entered and cross checked with relevant data recorded elsewhere in this booklet and in the phenotyping booklets for the mother of the affected woman and the index affected baby

Signed…………………………………………… **Date……………………**

Investigator/Research Assistant

7.6.6 Data collection form- Baby

Study number.....

A comparative study of viruses, bacteria and fungi present in blood, urine, amniotic fluid, and placental tissue of women with pre-eclampsia and normal pregnant women

PHENOTYPIC DATA
Baby of Pre-eclamptic or Normotensive pregnant woman

Screening Number | | | | | / | | | |

Name .....

Address .....

.....

.....

.....

Telephone number .....

Hospital number .....

Date of birth ...../...../.....

Data Protection and ConfidentialitAfter completion of this page, ensure that the subject study number is entered on all pages of this booklet. Then detach this page and store separately from the remainder of the booklet.

Screening Number ☐☐☐☐ / ☐☐☐

Gestational age at birth ☐☐ weeks ☐ days

Sex ☐ 0. Male

☐ 1. Female

Baby's weight in kilograms ☐.☐☐☐

Live birth ☐ 0. No ☐ 1. Yes

Apgar score at birth ☐☐ Record 1 to 10

Admitted to SCBU ☐ 0. No ☐ 1. Yes

COMMENTS

Record reasons for missing data and any additional relevant comment: ENSURE THAT ANONYMITY IS PRESERVED.

Signed..... Date.....

Investigator/ Research Assistant

7.7 PUBLICATIONS & ABSTRACTS PRESENTED

- **Amarasekara R**, Jayasekara RW, Senanayake H, Dissanayake VHW. "The detection of pathogenic bacteria present in the placental tissues of women with preeclampsia using novel molecular genetic techniques". Society for Gynaecology Investigations (SGI) summit. September 5-8, 2013. Istanbul, Turkey. (Selected among the top ten oral abstract for the SGI summit 2013, Turkey). Certificate enclosed

- **Amarasekara, R.**, Jayasekara, R.W., Senanayake, H., & Dissanayake, V.H.W. "Detection of pathogenic microbes in the placental tissues of women with preeclampsia using metagenomics technology." 46th Annual Scientific Sessions of the Sri Lanka College of Obstetricians and Gynaecologists. August 16-18, 2013. Colombo, Sri Lanka. (Abstracted in Sri Lanka Journal of Obstetrics and Gynecology, 2013; **35** (2): 40)

- **Amarasekara R**, Jayasekara RW, Senanayake H & Dissanayake VHW. "Association of Infectious Agents with Pre-eclampsia". Presented in Wellcome Trust Advanced Course: Molecular Basis of Bacterial Infection. Hinxton, Cambridge. May 12-18, 2013.

- **Amarasekara R**, Jayasekara RW, Senanayake H & Dissanayake VHW. "Association of Infective agents with Pre-eclampsia". 45th Annual Scientific Sessions of the Sri Lanka College of Obstetricians and Gynecologists. October 17 - 23, 2012. Colombo, Sri Lanka. (Abstracted in Sri Lanka Journal of Obstetrics and Gynecology, 2012; **34**: 69)

- **Amarasekara R**, Jayasekara RW, Senanayake H & Dissanayake VHW. "Complications associated with pre-eclampsia". 125th Anniversary International Medical Congress, Sri Lanka Medical Association. July 2 - 6, 2012. Colombo, Sri Lanka. (Abstracted in the Ceylon Medical Journal, 2012; **57**:96)

- **Amarasekara R**, Jayasekara RW, Senanayake H & Dissanayake VHW. "Complications associated with pre-eclampsia". Abstracted in 'Global Forum of Sri Lankan Scientists: Empowering Sri Lanka, National Science Foundation (NSF), Colombo, Sri Lanka. December 13-15, 2011.

- **Amarasekara R**, Jayasekara RW, Senanayake H & Dissanayake VHW. "Complications associated with pre-eclampsia". Submitted after revision to Journal of Obstetrics and Gynaecology (JOG).

KÖK HÜCRE VE GEN TEDAVİSİ DERNEĞİ
2005

sgi
"Science in the Service of Women's Health"

SGI SUMMIT TURKEY 2013

Innovations in Obstetrics and Gynecology

September 5 - 8, 2013
Harbiye Military Museum and Culture Center, Istanbul, Turkey
Society for Gynecologic Investigation

CERTIFICATE OF APPRECIATION

The oral abstract which is presented in SGI 2013 Turkey on 5-8 September 2013,
" The detection of pathogenic bacteria present in the placental tissues of women with pre-eclampsia suing novel molecular genetics technique"

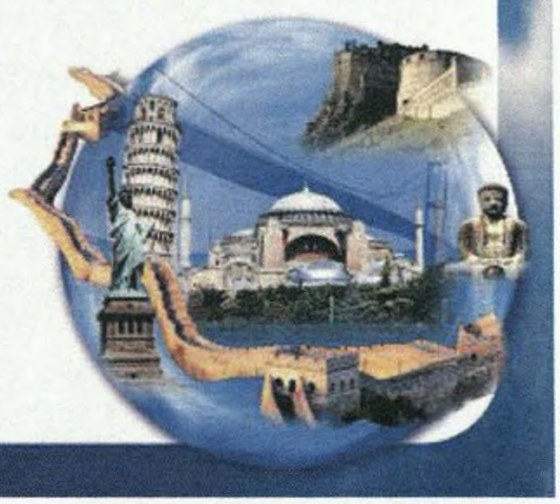
Amarasekara, R., Jayasekara R.W., Senanayake, H., Dissanayake, V.H.W

has been elected as one of top 10 oral abstract.

Summit President
Erkut Attar

Summit Honorary President
Serdar Bulun

Summit Scientific Secretary
Ercan Baştu



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