VAGINAL MICROBIOME IN WOMEN WITH PRETERM BIRTH AND THOSE WITH TERM BIRTH THAT ATTENDED ANC AT THIKA LEVEL 5 COUNTY REFERRAL HOSPITAL BETWEEN JANUARY 2019 AND MARCH 2019.

A case-control study

This thesis is submitted in partial fulfillment for the award of a Master of Medicine in Obstetrics and Gynecology at the College of Health Sciences, University of Nairobi.

Principal Investigator

Dr John Mwangi Kamau

MMED obstetrics and gynecology (UoN) Registration Number: H58/11238/2018

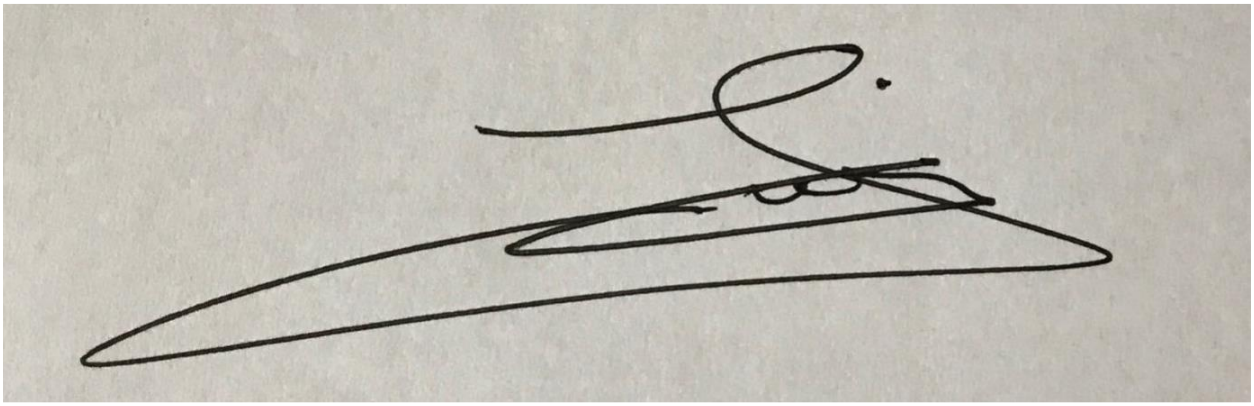
Email:jmkamau38@gmail.com

29th AUGUST 2022

DECLARATION

I declare that this thesis is my original work and has not been presented elsewhere for the award of a degree.

Date: 29th August 2022



Dr. John Mwangi Kamau

Registration number: H58/11238/2018

SUPERVISOR'S APPROVALS

This thesis has been submitted for examination with our approval as University Supervisors

Dr. Onesmus Gachuno, MBchB, MMed (Obs&Gyn), PGDRM

Senior Lecturer Department of Obstetrics and Gynaecology

Sign:

Date: 30th August 2022

Prof. Obimbo Moses Madadi (MBChB, Dip FELASA C, MSci, MMed (Obs&Gyn), PhD, Post Doc)

Senior Lecturer Department of Obstetrics and Gynaecology

Chair Department of Human Anatomy

Sign: 

Date: 30th August 2022

Prof. Omondi Ogutu. MBChB, MMed (Obs&Gyn,) PGDRM, FCOG (ECSA)

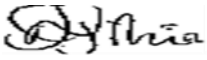
Associate Prof of Obstetrics and Gynaecology

Sign: 

Date: 30th August 2022

Dr. Jesse Gitaka. MBchB, MTropMed, PhD

Lecturer Mount Kenya University

Sign: 

Date: 30th August 2022

CERTIFICATE OF AUTHENTICITY

This is to certify that this thesis is the original work of Dr John Mwangi Kamau, registration number H58/11238/2018, an M.Med student in the Department of Obstetrics and Gynaecology, College of Health Sciences, University of Nairobi, under the guidance and supervision of Dr. Onesmus Gachuno, Prof. Moses Obimbo, Prof. Omondi Ogutu and Dr. Jesse Gitaka. This is to confirm that this proposal has not been presented in any other University for the Award of any other degree.

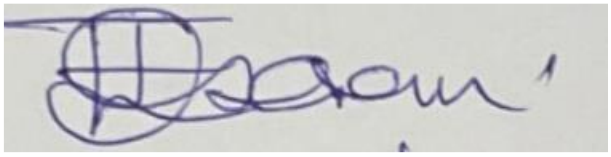
Professor Eunice Cheserem,

MBChB, MMed (Obs&Gyn), Dip. International maternal health care, PGD bio-medical research methodology.

Associate Professor and chair,

Department of Obstetrics and Gynaecology

University of Nairobi.

Signature: 

Date: 29th August 2022

DEDICATION

I dedicate this work to my lovely wife, Margret Wambui and our 3 children- Matthew, Janice and Malachi. Thank you for the moral support and your constant encouragement.

ACKNOWLEDGEMENT

This dissertation has been made possible by the great support of a big team to who am entirely thankful.

Thanks to the Almighty Lord for the strength and mind-set to perform this research.

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A special appreciation to the research team at Mount Kenya University for the support and lessons on metagenomic studies.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ABBREVIATIONS

1. ANC: antenatal clinic
2. BMI; body mass index
3. CCL 2: C-motif chemokine ligand 2
4. CST- community state type
5. Ha- alternate hypothesis
6. Ho- null hypothesis
7. HVS; high vaginal swab
8. IL: interleukin
9. MMP: Matrix metalloproteinase
10. PGF2α; prostaglandin F 2 alpha
11. PTB; preterm birth
12. TNFα; tumour necrosis factor alpha

Operational definitions

1. Vaginal microbiome- the collective genome of microorganisms inhabiting the vagina
2. Vaginal microbial- the collective microorganisms inhabiting the vagina

ABSTRACT

Background:

Vaginal microbiome influences pregnancy outcomes and the presence of vaginal dysbiosis has been shown to cause preterm birth. Approximately, there were 15 million preterm births per year, globally and 25% are attributable to vaginal dysbiosis. In Kenya, 1 in 6 under 5 deaths per year is attributable to preterm births.

Traditionally, microscopy and culture has been the cornerstone of establishing the vaginal microbial composition. In the recent decades, use of non-culture techniques such as metagenomics has been shown to be more specific and sensitive thus superior to culture based techniques. Metagenomic based studies have shown that vaginal microbiome varies by race, geographical location, ethnicity, and diet among other variables. Our study objective was to determine the differences in vaginal microbiome in women with preterm birth and those with term birth at Thika Level 5 Hospital, Kenya.

Methods:

In this comparative case-control study, we utilized bio-banked high vaginal swabs. We had 39 cases and 67 controls that were selected through purposive sampling. DNA extraction was done using DNeasy Qiagen extraction kit. Gene sequencing and taxonomic profiling was then done using NGS Miseq illumina platform at Macrogen Inc. South Korea.

Results:

Lactobacillus iners was the most common bacteria found making CST 3 the most abundant at 64% and 67% in the cases and controls respectively but was present in 90|% of cases and 84% of the controls. CST 4 abundance was 28% in both cases and controls while CST 5 was dominant in 8% of cases and 5% of controls. CST 1 and 2 were not recorded in this study. However, there was one case of *L. gasseri* among preterm cases though this was not abundant enough to classify a woman as CST 2. CST 4 was present in 80% of the cases and 67% of the controls. The differences in the cases and controls were not statistically significant.

Conclusion:

We confirmed the dominance of CST 3 and 4 in pregnant African women. The study also showed the co-existence of CST 3 and 4, however, there was no association between a specific CST and the risk of preterm.

Recommendations

Longitudinal sampling at different gestational ages up to 6 weeks postpartum would enable the establishment of the stability of the vaginal microbiome throughout pregnancy and changes

noted in the pueperium period. Cervical length is the best predictor of preterm, and since studies have shown that cervical length correlates to the diversity of the vaginal microbiome, future studies should involve both establishing the vaginal microbiome and cervical length simultaneously.

1. INTRODUCTION

Vaginal microbiome

The term vaginal microbiome is used to define the collective genome of the microorganisms in the vagina. These microorganisms have varying effects and based on their effect, they are classified as mutualistic, commensal or pathogenic(6). Mutualistic microbes are symbiotically beneficial and contribute to the physiology of the genital tract. The commensal microbes are neither harmful nor beneficial while the pathogenic microbes are harmful. This microbial composition is not static and it undergoes changes in response to physiological changes such as menstrual cycle and pregnancy. However, in pregnancy, the microbiome is stable and is characterized by a lactobacillus dominated community (7).

Preterm birth

This refers to delivery of a fetus before thirty seven completed weeks of gestational age. Worldwide, approximately 9-12% of pregnancies are delivered prematurely. This represents approximately 15 million preterm births per year(2). The highest rates are in Asia and sub Saharan Africa where the health care systems are week and given that prematurity increases the risk of morbidity and mortality, the strain on the health system is enormous. Consequently, prematurity is the number one cause of neonatal mortality and the second leading cause of under five deaths worldwide. Long term complications include; increased risk of cardiovascular disorders, neurodevelopmental disabilities, vision impairment, hearing impairment and learning difficulties. Overall, PTB is the main contributor of disability-adjusted life years(8).

Roberto et al., 2014; stipulated that preterm birth is a syndrome attributable to multiple pathologic processes. Such processes include intrauterine infections, low progesterone activity, disorders of the vascular system, placenta aging, uterine over-distension, pathologies of the cervix, and maternal-fetal intolerance. Of these, only infection has been proven and has been shown to cause approximately 25% of preterm births.

The understanding of risk factors and causes of preterm birth is vital in alleviating the burden of prematurity. Prematurity burdens the health care system due to the multitude of early and late complications. It also has huge socioeconomic effects on affected families. Thus prevention of preterm delivery is crucial and it is also in line with the targets of the third sustainable development goal which is on ensuring healthy lives and promoting well-being for all at all ages.

One of the targets is by 2030; the world will have reduced neonatal mortality to below 12 per 1000 live births and under-five mortality to less than 25 per 1000 live births.

Term birth

This refers to delivery of a fetus after thirty seven (37) completed weeks of gestational age. Studies have shown that delivery at term is essential for optimal neonatal outcome(9). To enable delivery at term several maternal and fetal characteristics have been identified. They include; non-extreme age, appropriate BMI, lack of substance abuse, singleton pregnancies, lack of medical co-morbidities, a history of previous term births and attendance of prenatal clinic(7).

Effect of vaginal microbiome on the timing of delivery

Vaginal dysbiosis is associated with spontaneous preterm births and approximately 25-30% of PTBs are attributable to intrauterine infection(1). The main route of intrauterine infection is an ascending infection from the vagina, and cervix. Organisms that have been isolated from fetal membrane of premature neonates are those in the vaginal vault. The most common organisms include; mycoplasma, ureaplasma, candida and group B streptococcus. Notably, Lactobacillus iners is a risk factor for preterm births despite it being part of the symbiotic vaginal flora(10).

These effects of vaginal microbiome on timing of delivery and complications of preterm births, necessitates the need to understand vaginal microbiome further. Non-culture based methods enable the understanding of which microbiomes and at what level of abundance are essential in enabling a conducive environment for term births. This study aims to fill this knowledge gap in our region.

1.1. LITERATURE REVIEW

1.1.1. Vaginal microbiome

The term vaginal microbiome refers to the collective genome of microbes inhabiting the vagina. These microbes contribute to the physiology of the female genital tract through various ways such as providing resistance to pathologic infections, breaking down nutrients and educating the immune system. They are also a source of pioneer bacteria in the neonate's gut where it plays a crucial role in educating the immune system of the neonate. Vaginal flora composition is varied. This variation is dependent on various factors such as genetic make-up, dietary intake, reproductive cycle stage and disease state. However, during pregnancy, the composition becomes more stable as gestation progresses. The stability during pregnancies is attributable to several factors such as; the lack of cyclic hormonal fluctuations and menstrual flow in pregnancy, the absence of alterations in cervical and vaginal secretions during pregnancy and the reduced sexual activity as gestation progresses(11). This stability plays a crucial role in prevention of advance pregnancy outcomes(12).

In pregnancy, the dominant vaginal microbiome is lactobacillus. Lactobacillus inhibits pathogenic bacterial growth through secretions of antibacterial bacteriocins and the production of metabolites such as lactic acid to maintain a low, hostile Ph. (7). Other species that may dominate the vaginal microbiome in pregnancy include clostridiales, bacteroides, and actinomycetes. Currently, with the use of next generation gene sequencing, many other species that form part of the vaginal microbiome have been identified including novel microorganisms (6).

The vaginal microbiome is studied under five community state types (CST). CST 1 is Lactobacillus crispatus dominant, CST 2 is Lactobacillus gasseri dominant, CST 3 is Lactobacillus iners dominant, CST 4 is Prevotella, Dialister, Atopobium vaginae, Gardnerella vaginalis, Megasphaera, Peptoniphilus, Sneathia, Finegoldia, and Mobiluncus dominant, and CST 5 is Lactobacillus jensenii dominant. Caucasians have a dominance of CST 1 and 5 while Asians have a dominance of CST 2 and 5. Africans have a dominance of CST 3 and 4. The dominance of CST 4 increases the risk of bacterial vaginosis, thus Africans are at an increased risk of bacterial vaginosis and thus preterm births(7).

1.1.2. Pre-term births and characteristics of patients with preterm births.

The WHO defines preterm birth as delivery of a fetus before thirty seven (37) completed weeks of gestation age. It is further divided into three sub-categories namely;

a) Extremely-preterm which is delivery from 20 weeks to twenty seven (27) completed weeks of gestation age.
b) Very-preterm which is delivery between twenty eight (28) to thirty one (31) weeks and six (6) days of gestation age.
c) Late-preterm which is delivery between thirty two (32) weeks to thirty six (36) weeks and six (6) days of gestation age.

Worldwide, 9-12% of all pregnancies are delivered before term representing approximately fourteen (12) million preterm births per year with the highest rates of preterm births being in Asia and sub-Saharan Africa. The rate of preterm births per year in sub Saharan Africa is 8.6-16.7%. Sub Saharan Africa contributes to approximately 28% of the global preterm births; this represents approximately 4.2 million preterm births per year(2). Locally, the rate of preterm births is estimates at 12.3 % of all pregnancies per year(9). Globally, preterm birth is the number one cause of neonatal morbidity and mortality. In Kenya, 1 in 6 under-5 deaths per year are related to preterm births. This underscores the burden of preterm births on the health system.

Two thirds of preterm births occur spontaneously and the rest are medically induced. Medical induction is done due to or to prevent worsening of maternal complications or fetal complications. Maternal complications that can lead to induction of preterm delivery include eclampsia, severe pre eclampsia, and uncontrolled cardiac disease in pregnancy among others.

Fetal complications that may lead to induction of preterm delivery include intra-uterine growth restriction, twin to twin transfusion, and anencephaly among other conditions.

Romero R. et al., 2014; postulated that preterm birth is a syndrome attributable to multiple pathological processes. They classified these processes into ten groups namely , infection, vascular disorder, decidual senescence, uterine over distension, decreased progesterone levels, cervical disease, breakdown of maternal-fetal tolerance, stress and unknown pathological processes. Among these pathological processes, only infection has been proven to cause preterm birth. This underscore the need to identify which microbes cause preterm birth and which ones are essential for providing a conducive environment for term birth.

Prematurity is associated with multiple complications for both the mother and the neonate. Neonatal complication can be short term and long term. The short term complications are mainly due to immaturity of multiple organ systems. They include acute respiratory distress, apnea, and hypotension, patency of ductus arteriosus, increased risk for necrotizing enterocolitis, gastroesophageal regurgitation, and increased risk of infection among others. Long term complications include neurodevelopmental disorders such as cerebral palsy, intellectual disabilities, visual impairment and hearing impairment among others.

Several studies have been conducted to predict the risk of preterm birth. Currently, cervical length and a history of previous preterm birth are known predictors of preterm delivery. Several patient characteristics have also been shown to influence the rate of preterm birth. These characteristics include: race, age, BMI, substance use, medical co-morbidities, multiple gestations, history of preterm birth, and socioeconomic status. Romero R. et al., 2014; established that the rate of preterm birth among Africa-Americans to be higher (16-18%) than among the Caucasians (5-9%). These findings have been similarly found to be true in studies done in Europe. Extremes of maternal age, multiple gestations, low BMI and obesity increase the risk of preterm birth. Maternal co-morbidities such as hypertension, diabetes mellitus, asthma, and thyroid disease also increase the risk of preterm birth.

1.1.3. Term births and characteristics of patients with term births

Term birth refers to delivery of a fetus after thirty seven (37) completed weeks of gestational age(13). It is further subdivided into early term and full term. Early term refers to deliveries between thirty seven (37) weeks to thirty eight (38) weeks and six (6) days of gestational age. Full term refers to deliveries between thirty nine (39) weeks to forty one (41) weeks and 6 days of gestational age. Deliveries after forty one (41) weeks and six (6) days are referred to as post term births.

Fleischmer AR et al., 2010; established that early term neonates have an increased mortality and morbidity rate as compared to full term neonates. The neonatal mortality rate at early term was 0.66 per 1000 live births while in full term 0.33 per 1000 live births. Thus the designation of

'term' carries a significant clinical implication in the management of pregnancy complications and elective deliveries.

Patient characteristics that have shown to influence the rate of term births include; race, non-extreme age, appropriate BMI, lack of substance abuse, singleton pregnancies, lack of medical co-morbidities, history of previous term births and attendance of prenatal clinic.

1.1.4. Relationship between the vaginal microbiome and the timing of delivery

The stability, diversity, and abundance of vaginal microbiome correlate to the timing of birth. A pregnancy driven stronghold on the vaginal microbiome has to be maintained throughout the pregnancy to prevent preterm birth. This stronghold enables the dominance of mutualistic and commensal microbials that are essential for term delivery. Cervical length, a key predictor of preterm birth, also correlates to the diversity and abundance of vaginal microbiome (12).

Bacterial vaginosis has been shown to increase the risk of preterm births five folds(14). However, even in the absence of vaginal dysbiosis, the presence or absence of specific microbiome in the vaginal has been associated with higher rates of preterm birth. It is thus important to identify the vaginal microbiomes of pregnancy and classify them as either mutualistic or commensal microbiota as opposed to pathogenic microbiota. The classification should also take into account the abundance of the microbe as related to its effect on the pregnancy. This is because some mutualistic and commensal microbes trigger birth depending on how abundant they are in the vagina. For example, lactobacillus iners triggers birth when more abundant(15).

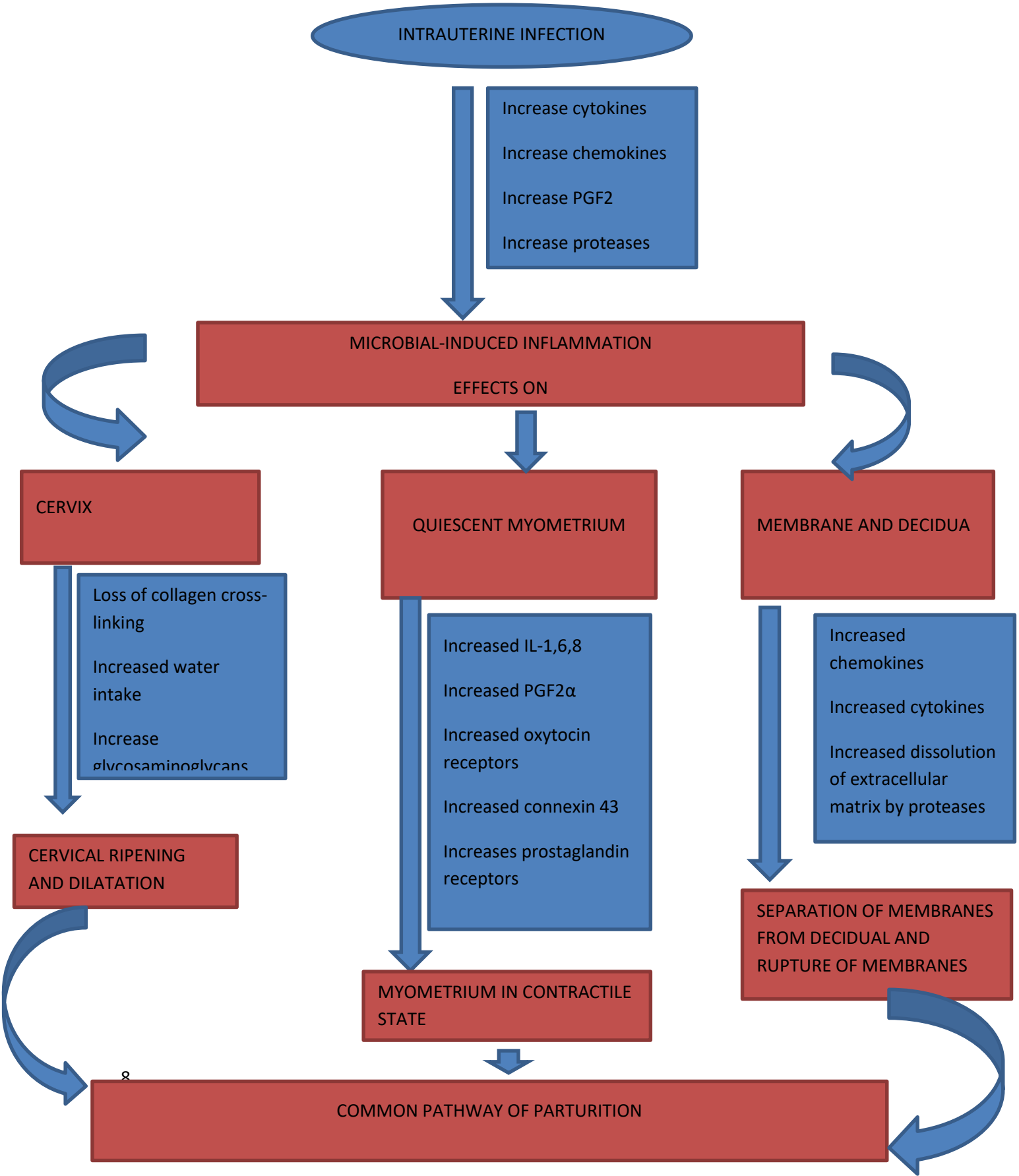
Birth, whether term or preterm, involves similar clinical events that lead to increased uterine contraction, cervical dilatation and rupture of membranes. This results in the expulsion of the fetus, fetal membranes and placenta. Intra amniotic infections have been shown to lead to birth. About 25% of PTB have been shown to have intra amniotic infections. There are three hypothesized mechanisms on how these bacteria seed into the fetoplacental unit(16).

a) Genitourinary-fetoplacental route. The microorganisms ascend from the vaginal and cervical mucosa into the fetoplacental unit. This is the most common route.
b) Gastrointestinal-fetoplacental route. The maternal dendritic cells internalize and transport the microorganisms into the fetoplacental unit.
c) Oral-fetoplacental route. Oral microbiome disseminate via bloodstream into the fetoplacental unit.

The presence of microorganisms in the fetoplacental unit is sensed by pattern recognition receptors such as toll-like receptors (TLRs). This induces a pro-inflammatory response that involves the production of inflammatory chemokines and cytokines such as IL 1, 6, 8, CCL 2, TNF α. Production of PGF2α, proteases such as MMP 8, MMP 9 and contraction associated proteins such as oxytocin receptors and connexin 43 is also increased. However, production of progesterone is reduced. These changes affect the cervix, myometrium and chorioamniotic

membrane. At the cervix, these inflammatory mediators cause loss of collagen cross-linking, and increased water intake. This leads to cervical ripening and dilatation. At the myometrium, they cause a shift from a quiescent state to a contractile state. The proteases also cause dissolution of extracellular matrix at the chorioamniotic-decidua region leading to separation of the chorioamniotic membrane from decidua and eventual rupture of membranes. These changes lead to a common pathway of parturition involving cervical dilatation, uterine contraction and expulsion of the fetus and membranes.

Diagrammatic Illustration on how infections trigger birth



1.1.5. Role of vaginal microbiome metagenomics in unveiling pathogens causing adverse pregnancy outcomes

Vaginal microbiome may be classified depending on their effect as mutualistic, commensal or pathogenic. The effects of these microbiomes depend on the type and level of abundance of the species as well as the physiological state of the host(17). Traditional culture-based methods only identify the known species and have lower sensitivity and specificity as compared to culture-independent methods. Culture-based methods also do not quantify the level of abundance of the species. The sensitivity and specificity of metagenomics is 99% and 100% respectively. Vaginal microbiome metagenomics enables the identification of all genomes present in the habitat on study. These may include novel microorganisms that would otherwise have been missed by culture-based methods. Determining the levels of abundance of mutualistic microbes that are essential for term pregnancy is crucial and guides the role of probiotics in pregnancy. Metagenomics also aides to identify the level of abundance at which commensal microbiomes may become pathogenic, something that culture based methods cannot establish.

1.1.6. METAGENOMICS

Metagenomics is a non-culture based technique used to study the collective community of microorganisms that inhabit a particular environment(18). It enables the identification of genomes of those microorganisms in that particular environment. Traditional culture-based techniques have several disadvantages such as being birth intensive, time consuming and in a polymicrobial field, they fail to identify the abundance of each microorganism. They also require identification of know features in an organism for identification thus cannot identify new organisms or organism that have undergone evolutionary change. The use of culture-independent techniques increases the identification of more microbiome, defines the abundance of each microbiome in the specific environment and unlike PCR-based methods, metagenomics enables the identification of novel microorganisms. Steps involved in metagenomics include, sample collection and preservation, DNA extraction, Gene sequencing and metagenomic profiling(18).

1.1.6.1. Sample collection and preservation

The sample should be collected in a sterile procedure as the instruments and reagents used may contain microbial that contaminate it. These instruments and reagents must also be properly documented as this information will be considered during gene analysis and validation. If the sample collection is done in an environment in which the microbial under study are scarce, several enrichment techniques are used to improve accuracy. The laid down preservation protocols must be adhered to ask the outcome may be affected by time between collection and preservation. The number of freeze-thaw cycles also affects the outcomes.

1.1.6.2. DNA extraction

The choice on the method of DNA extraction is a balance between its lysing-diversity and its level of DNA fragmentation. The method of should lyse a diverse group of microbial thus avoiding the bias to derive easy-to-lyse microbial only. Mechanical lysing methods like bead-beating, lyse more diverse microbial but cause more DNA fragmentation. While chemical lysing methods are less lyse-diverse but cause less DNA fragmentation. The DNA fragmentation affects gene sequencing and metagenomic profiling because the number of reads obtained is dependent on genome size, within-species heterogenicity, and relative abundance of species.

1.1.6.3. Gene sequencing

Sequencing platforms for metagenomics can be broadly classified into chain-terminator sequencing and next generation sequencing. The chain-terminator (Sanger) sequencing is easy to use and reliable but it has a low throughput and is biologically biased. The biological bias is because it requires cloning of foreign DNA into a vector. High throughput sequencing is essential due to microbial diversity and non-uniform abundance. Next generation sequencing techniques are cheaper, have a high throughput, and have no biological bias as there is no cloning required. Next generation sequencing techniques include; illumina sequencing, roche 454 sequencing and ion torrent personal genome machine. This study will use Illumina sequencing.

1.1.6.4. Metagenomic profiling

Metagenomic profiling can be assembly based or assembly-free. Assembly-free or mapping metagenomic profiling identifies microbial species in a genome and estimates their abundance by mapping the reads on external sequence data resources. The mapping can be against the genome (taxonomic profiling) or against a functional protein or pathway (metabolic profiling). The main disadvantage of mapping is that it cannot identify a genome that is not in the external sequence data resource used. This can be mitigated by choosing a reference genome with a high diversity. This study will utilize NGS Miseq illumina platform at Macrogen.

PROBLEM STATEMENT

Difference in vaginal microbiome and local immune response of the vagina between women who develop spontaneous preterm birth and those who have spontaneous birth at term remains unanswered. This study shall try and answer whether the vaginal microbiome is different among these two groups thus contributing to knowledge advancement.

Locally, studies on vaginal microflora have utilized microscopy and culture-based techniques. This method of bacteria identification requires a comparison of the morphology, growth requirement, and enzymatic action to the reference strains. These factors can be altered by stress or evolution thus leading to misidentification of the bacteria. The bias and inexperience of the microbiologist can also lead to misidentification of the bacteria. Culture-based methods also

have a long turnaround time. This study will be a culture-independent based study thus eliminating the above shortfalls of culture based methods.

Metagenomic methods of study are objective, reliable and accurate. They have a sensitivity of 99%-100% and a specificity of 100% [4]. The sensitivity depends on three factors;

1. The amount of nucleic acid available for detection. The threshold is 1-5% relative abundance.
2. The ratio of target microbial nucleic acid to the host's nucleic acid.
3. The ratio of target to non-target microbial nucleic acid.

In this study, the above factors will be met to ensure maximum sensitivity and specificity.

STUDY JUSTIFICATION

The relationship between the mucosa of the lower genital tract and the microbial ecosystem plays a vital role as to why some women develop an ascending intra-amniotic infection while others do not. The microbial ecosystem also plays a key role in determining the cervical length. Since vaginal dysbiosis and cervical length are predictors of spontaneous preterm birth, research on vaginal microbiome is vital. Such research enables the understanding of vaginal microbiome as being mutualistic, commensal, or pathogenic. Since some species are either commensal or pathogenic depending on their level of abundance, such research should also define abundance of each species identified.

Previous studies have used the Nugent score to diagnose normal or disturbed vaginal microbiota. This score relies on gram stains of vaginal smears. It considers the number of cells per field of lactobacillus, bacteroides or gardnerella and mobiluncus. Each is given a score of 0-4. A score of +1 is given when there is less than 1 cell per field, +2 for 1-5 cells per field, +3 for 6-30 cells per field and +4 for more than 30 cells per field. Lactobacillus scores between 0-4 and so does bacteroides/gardnerella but mobiluncus scores 0-2. The scores are summed up and interpreted as normal (0-3), intermediate bacterial count (4-6) and bacterial vaginosis (7-10).

This scoring system fails to account for the many other species of vaginal microbiome and the various subspecies of these microbiomes. This study seeks to use next generation gene sequencing technique to determine the wide array of vaginal microbiome and their abundance level in preterm and term births. The use of 16S rRNA gene sequencing will enable us to capture all bacteria and fungi in the vaginal with a close to 100% specificity and sensitivity.

The determination of differences in vaginal microbiome in women with preterm birth and those with term birth shall be of value in the management of pregnant women. For example, choosing an antibiotic that does not disrupt the vaginal microbiome and providing guidance in the

formulation of vaginal probiotic supplements. Since the vaginal microbiome is dependent on various factors among them race, geographical location and ethnicity, a study within our population will be of great value.

HYPOTHESIS

Ho- Among women who attended ANC at Thika level 5 County Referral Hospital between January 2019 and March 2019, there are no differences in the vaginal microbiome of women who had preterm birth and those who had term birth.

Ha- Among women who attended ANC at Thika level 5 County Referral Hospital between January 2019 and March 2019, there are differences in the vaginal microbiome of women who had preterm birth and those who had term birth.

RESEARCH QUESTION

Are there differences in the vaginal microbiome in women with preterm birth and those with term birth that attended ANC at Thika level 5 county referral hospital between January 2019 and March 2019?

BROAD OBJECTIVE

To determine the differences in the vaginal microbiome in women with preterm birth and those with term birth that attended ANC at Thika level 5 county referral hospital between January 2019 and March 2019.

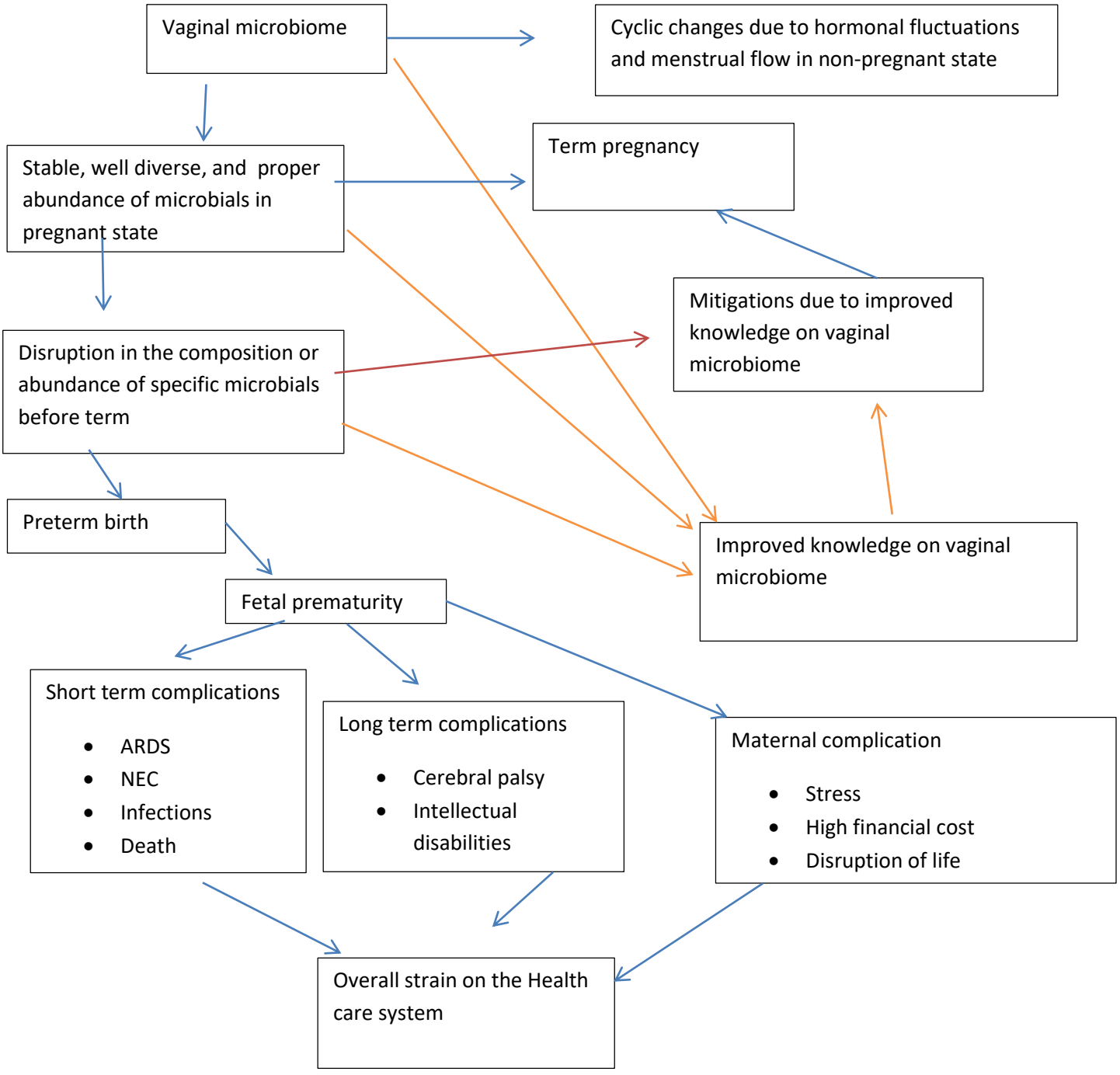
SPECIFIC OBJECTIVES

In women with preterm birth and term birth who attended ANC at Thika level 5 County Referral Hospital between January 2019 and March 2019;

a) Use metagenomics to determine their vaginal microbiome.
b) Compare and analyze the vaginal microbiome.
c) Determine their socio-demographic characteristics.

CONCEPTUAL FRAMEWORK

The study used metagenomics to determine the vaginal microbiome. It is these microbes that may ascend from the vaginal and cervix into the intrauterine space causing an intrauterine infection that triggers birth. The triggering of birth due to intrauterine infection may occur even before term


Vaginal microbiome
Cyclic changes due to hormonal fluctuations and menstrual flow in non-pregnant state
Stable, well diverse, and proper abundance of microbials in pregnant state
Term pregnancy
Mitigations due to improved knowledge on vaginal microbiome
Disruption in the composition or abundance of specific microbials before term
Preterm birth
Improved knowledge on vaginal microbiome
Fetal prematurity
Short term complications
ARDS
NEC
Infections
Death
Long term complications
Cerebral palsy
Intellectual disabilities
Maternal complication
Stress
High financial cost
Disruption of life
Overall strain on the Health care system

2. METHODOLOGY

2.1 Study design

A case-control study

2.1. Study site and setting

The primary data was collected for an ongoing study titled 'rapid and multiplex diagnosis of maternal bacterial infection'. The data was collected at Thika level 5 County Referral Hospital in Kiambu County, Kenya. It was collected in the third trimester from women with different medical conditions and complications. The women were then followed up until delivery. The specimens were bio-banked at the Basic Clinical and Translational laboratory in Chiromo and KAVI institute of clinical research laboratory.

2.2. Study population

Pregnant women who attended ANC at Thika level 5 County Referral Hospital between January 2019 and March 2019

2.3. Inclusion criteria

Pregnant women who attended ANC at Thika level 5 County Referral Hospital between January 2019 and March 2019 and

1. Were available for follow up until delivery at the facility
2. Provided informed consent for the primary study.
3. Had a singleton pregnancy
4. Had spontaneous onset of birth

The cases will be those that delivered at or before 36 weeks and 6 days, while the control will be those that delivered between 37 weeks and 41 week plus 6 days.

2.4. Exclusion criteria

Pregnant women who attended ANC at Thika level 5 County Referral Hospital between January 2019 and March 2019 but;

a) Had used antibiotics within a week prior to inclusion as this interferes with the vaginal microbials
b) Had used vaginal antimicrobial pessaries or douched within a week prior to inclusion as this alters the vaginal flora
c) Had vaginal bleeding or documented placenta previa

d) Had chronic medical conditions such as CKD, DM, HIV, thyroid disease, hypertension, anaemia
e) Had multiple gestations as this is a risk factor for preterm birth.
f) Had documented uterine anomalies such as uterine septate
g) Had a history of cervical insufficiency
h) Were on immunosuppressive therapy

2.5. Sample size

The formula for estimation of sample size for case-control studies

$$\underline{\mathbf{n}} = \frac{r+1}{1}\frac{P(1-P)(Z\beta+z\frac{\alpha}{2})^2}{(p1-p2)^2}$$

where:

n is the sample size

r is the ratio of control to cases (ratio of controls to cases is 2:1)

P is the average proportion exposure calculated as (proportion of exposed cases + proportion of exposed control)/2

Zβ is the standard normal deviation for power. For 80% power, we use 0.84

Zα/2 is the standard normal deviation for a level of significance. For a level of significance of 0.05 we use 1.96

P1 is the proportion in cases while P2 is the proportion in controls. Therefore, (p1-p2) represents the effect size or difference in proportion expected based on previous studies.

Varkha Agrawala and Emmet Hirsch 2012 (19) established that 40% of preterm births are associated with intra-uterine infections while Seong Hyo Suk et al., 2008 (20) established that 13% of term pregnancies in active birth and un-ruptured membranes, have evidence of intrauterine infections. Thus, we shall use 0.40 as proportion of exposed cases and 0.13 as proportion of exposed controls.

This gives a P of (0.40+0.13)/2 = 0.265

$$\underline{\mathbf{n}}(\text{controls}) = \frac{r+1}{1}\frac{P(1-P)(Z\beta+z\frac{\alpha}{2})^2}{(p1-p2)^2} = \frac{2+1}{1}\frac{0.265(1-0.265)(0.84+1.96)^2}{(0.40-0.13)^2} = 70$$

since the ratio is 2:1, the cases will be 35

We add a 10% margin for errors in data collection and management.

This gives a total of 39 cases and 77 controls.

The primary study had a total of 233 participants.

2.6. Sampling procedure

Clinical data and high vaginal swabs were collected at Thika level 5 county referral hospital by trained health providers under the supervision of the principal investigators. Clinical data included the age, gestational age, BMI, and relevant medical history.

Upon being given informed consent, trained midwives collected swabs from the posterior vaginal vault and transported in Amies transport medium to the laboratory as soon as possible. The mothers were then followed up to delivery and the gestational age at delivery recorded. The swabs were bio-banked at The Basic Clinical and Translational laboratory in Chiromo and Kenya AIDS vaccine initiative ICR laboratory.

It is these samples that we purposively sampled for use.

2.7. Data variables

Table 1: Data Variables

Specific objective	Exposure variable	Outcome variable	Source of data
To determine the composition of vaginal microbiome in women with preterm births	CST present CST absent	birth before 37 weeks of gestational age	HVS for metagenomics
To determine the composition of vaginal microbiome in women with term births	CST present CST absent	Birth at term	HVS for metagenomics
To compare and analyze vaginal microbiome of women with preterm births to those with term births	CST present CST absent	timing of delivery	Statistical analyses of genome data in the two groups
To determine the socio-demographic characteristics of women with preterm birth	Age, BMI, parity, previous pregnancy outcome.	Birth before 37weeks of gestational age.	Clinical data collected in the questionnaire
To determine the socio-demographic characteristics of women with term births	Age, BMI, parity, previous pregnancy outcome.	Birth at term	Clinical data collected in the questionnaire

2.8. Data collection and management

The data on patient characteristics were recorded by the investigators using the prepared questionnaire. A high vaginal swab was also collected at a gestational age of between 20 weeks and 28weeks. The swabs were transported in Amies transport medium and stored as soon as possible. The patients were then followed up until delivery. The data and specimens are stored at The Basic Clinical and Translational laboratory in Chiromo and Kenya AIDS vaccine initiative ICR laboratory.

2.9. Determining vaginal microbiome using next generation gene sequencing

2.10. DNA extraction protocol

DNA extraction was done from high vaginal swabs in a sterile environment. DNeasy Blood and Tissue Kit (Qiagen) will be used to extract DNA according to the manufacturer's protocol from both the study and the control groups. An extract of the protocol is attached as appendix 1.

2.1.1. PCR and Illumina sequencing protocol

16S rRNA gene sequencing was done using standard PCR and Illumina MiSeq (San Diego, CA) protocols. Thermocycling will be initiated by a 5-minute incubation at 95°C. Cycling parameters will be 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 120 seconds. Products will then be diluted 1:15 in nuclease-free water (Promega). Amplification and sequencing of the V4 region of the 16S rRNA gene will be performed at the Kenya Aids Vaccine Initiative (KAVI) with the use of the dual indexing sequencing strategy developed by Kozich et al.,. 2013. Sequencing will be performed on the Illumina MiSeq platform, with a MiSeq Reagent Kit V2 (500-cycle format; MS102-2003; Illumina), according to the manufacturer's instructions with modifications found in Kozich et al., 2013. Each PCR reaction (20 µL) will contain 1.0 µM of each primer, 2.5 µL template DNA, 0.15 µL AccuPrime HiFi Polymerase, and DNase-free water to produce a final volume of 20 µL. PCR will be performed under the following conditions: 95°C for 2 minutes, followed by 30 cycles at 95°C for 20 seconds, 55°C for 30 seconds, and 72°C for 5 minutes, with an additional elongation at 72°C for 10 minutes. Sequencing libraries will be prepared according to Illumina's protocol for Preparing Libraries for Sequencing on the MiSeq (15039740 Rev. D) for 2 nM or 4 nM libraries. FASTQ files generated will be paired end reads.

2.1.2. Processing of 16*S* rRNA gene sequence data

QIIME, an open-source bioinformatics pipeline for performing microbiome analysis was used to assemble paired-read contiguous sequences, to trim, filter, and align sequences, to identify and remove chimeras, to assign sequences to bacterial taxonomies, and to cluster sequences into operational taxonomic units (OTUs) based on the percentage of nucleotide similarity (97% and 99%).

2.11. Research ethics

Ethical approval for the primary collection of samples was granted to Dr. Jesse Gitaka the PI and Prof. Obimbo Moses the co-investigator by Mount Kenya University, ethical and research committee. A copy is attached appendix 3.

Ethical approval to utilize those samples for this study was obtained from KNH-UoN Ethics and Research Committee. A copy is attached appendix 4

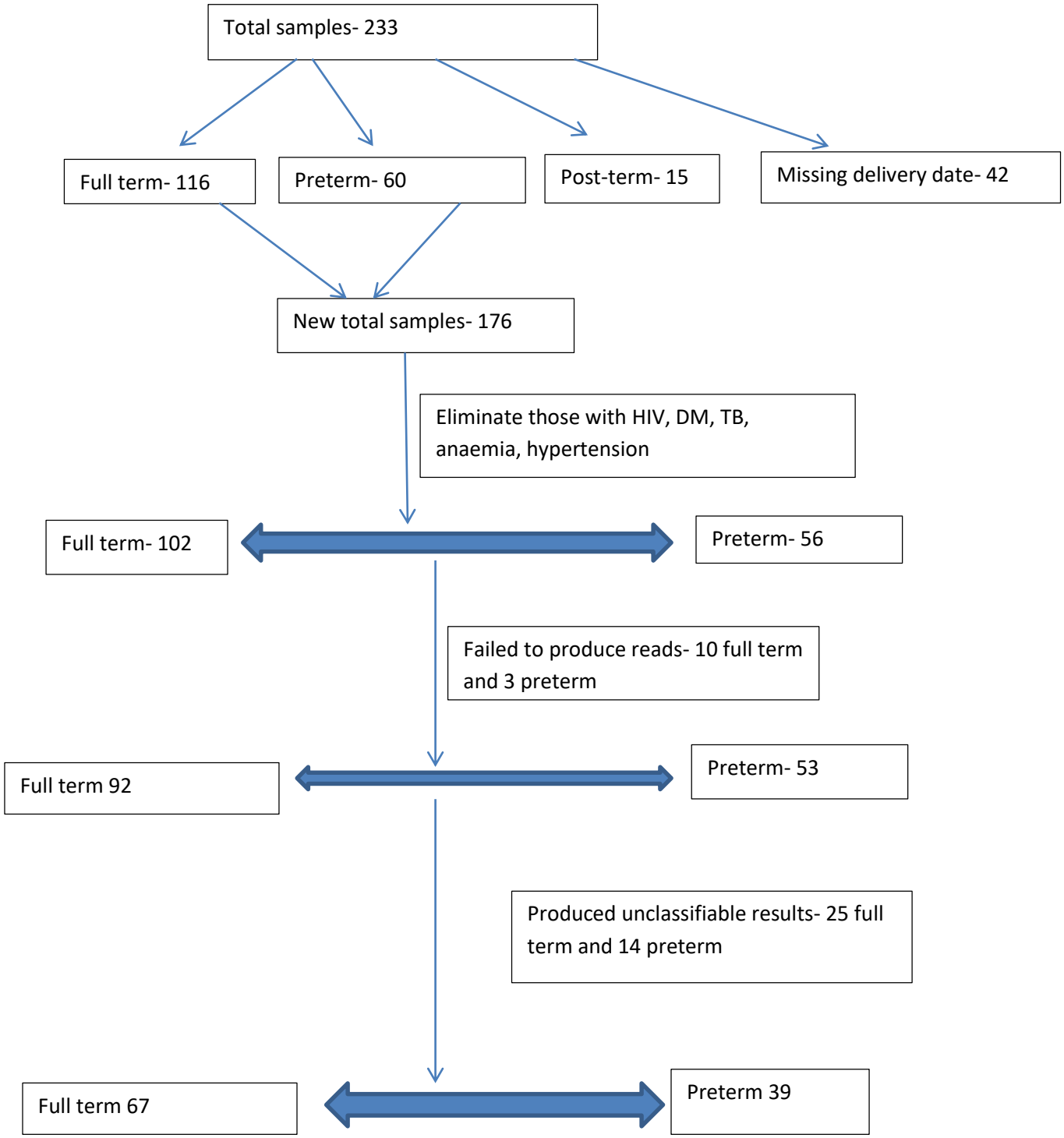
2.12. Statistical analysis

The socio-demographic and clinical characteristics were extracted from the client information form and recorded on the data extraction forms. Microsoft Excel was used for data entry. The data was then transferred to SPSS version 26 for cleaning and analysis.

Descriptive statistics were used to tabulate the characteristics of women with term and preterm birth. Mean and standard deviation for continuous variables were calculated and tabulated. The sociodemographic and clinical characteristics were compared with the timing of delivery (preterm and term) using t-test for continuous variables and chi-square/ Fisher's exact test for categorical variables. The vaginal microbiome community state types determined through QIIME were summarized with regards to the timing of delivery and chi-square test used to determine if there was any difference between the 2 groups. Other species observed were also summarized as frequencies and percentages. Finally, the CSTs were tabulated and compared to other Patient characteristics (age, parity, and gestation age) with Chi-square test of association.

For all statistical tests a p-value < 0.05 was taken to show statistical significance.

2.13. Study flow



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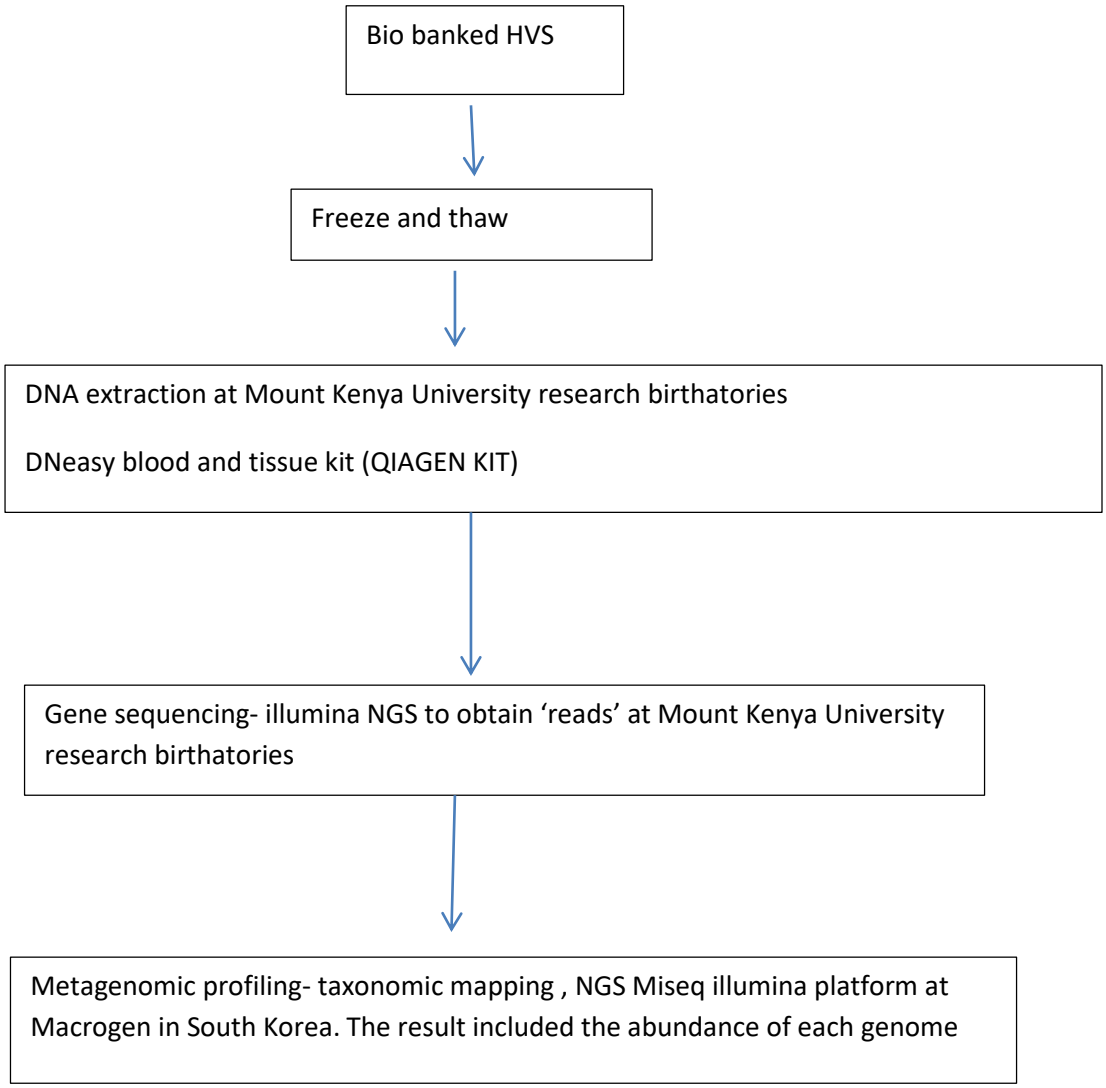
Bio banked HVS
Freeze and thaw
DNA extraction at Mount Kenya University research birthatories
DNeasy blood and tissue kit (QIAGEN KIT)
Gene sequencing- illumina NGS to obtain 'reads' at Mount Kenya University research birthatories
Metagenomic profiling- taxonomic mapping , NGS Miseq illumina platform at Macrogen in South Korea. The result included the abundance of each genome

3. RESULTS

3.1 Vaginal microbiome

Lactobacillus iners was the most common bacteria found making CST 3 the most abundant at 64% and 67% in the cases and controls respectively but was present in 90|% of cases and 84% of the controls. CST 4 abundance was 28% in both cases and controls while CST 5 was dominant in 8% of cases and 5% of controls. CST 1 and 2 were not recorded in this study. However, there was one case of L. gasseri among preterm cases though this was not abundant enough to classify a woman as CST 2. CST 4 was present in 80% of the cases and 67% of the controls. The differences in the cases and controls were not statistically significant. Table 2 below summarizes the findings of vaginal microbiome and timing of delivery.

Table 2: vaginal microbiome

		PRETERM **N=39**		TERM **N=67**		
		n	***(%)***	***n***	***(%)***	***p-value***
CST	3	25	64%	45	67%	0.833
	4	11	28%	19	28%	
	5	3	8%	3	5%	
L. crispatus (CST 1)	Absent	39	100%	67	100%	-
L. gasseri (CST 2)	Absent	38	97%	67	100%	0.368
	Present	1	3%	0	0%	
L. iners (CST3)	Absent	4	10%	11	16%	0.380
	Present	35	90%	56	84%	
CST 4	Absent	8	21%	22	33%	0.174
	Present	31	80%	45	67%	
L. jensenii (CST5)	Absent	36	92%	64	96%	0.667
	Present	3	8%	3	5%	

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3.2 Sociodemographic characteristics

The mean age of patients was 25 years and 26 years for the cases and controls respectively. The patients were mostly young (18-27), had middle income, were married and from urban areas with secondary education. This was however not statistically significant between the cases and controls. Table 3 summarizes the sociodemographic characteristics

Table 3:sociodemographic

		PRETERM N=39		TERM N=67		
						p-value
Age (years)		25	6.69 S.D	26	5 S.D	0.110
	18-27	28	72%	43	64%	0.110
	28-37	8	21%	23	34%	
	38-47	3	8%	1	2%	
Parity						
	Primipara	17	44%	30	45%	0.509
	Multipara	21	54%	37	55%	
	Grand Multipara	1	3%	0	0%	
Marital status	Single	11	28%	10	15%	0.098
	Married	28	72%	57	85%	
Level of education	Primary	6	15%	8	12%	0.977
	Secondary	17	44%	29	43%	
	Tertiary	16	41%	30	45%	
Income	<10000	3	8%	3	5%	0.575
	10000-30000	15	39%	19	28%	
	30000-50000	18	46%	37	55%	
	50000-100000	3	8%	8	12%	
Environment	Urban	39	100%	65	97%	0.53
	Rural	0	0%	2	3%	
Form of	YCS	11	28%	26	39%	0.270
	SVD	28	72%	41	61%	

CHAPTER 4

4.1 Discussion

Our study was able to compare the vaginal microbiome in women delivering preterm (cases) and those delivering at term (controls). The vaginal microbiome is generally classified into five Community State Types (CST) based on the presence and relative abundance of Lactobacillus species as follow; CST 1 where lactobacilli *crispatus* is abundance; CST 2 where Lactobacillus *gasseri* is abundant; CST 3 where lactobacillus *Iners* is abundant; CST 4 where there is an abundance of facultative anaerobes and lastly CST 5 where there is an abundance of lactobacillus *Jensenii* (Ravel et al.,, see table below). In our study, we studied both the presence and abundance and gave percentages of both.

Table 4:community state types

Community state type (CST)	**Dominant species**
CST 1	Lactobacillus crispatus
CST 2	Lactobacillus gasseri
CST 3	Lactobacillus iners
CST 4	Prevotella, Dialister, Atopobium vaginae, Gardnerella vaginalis, Megasphaera, Peptoniphilus, Sneathia, Finegoldia, and Mobiluncus
CST 5	Lactobacillus jensenii

In concurrence with other studies (David A.M et al., 2015 and Freitas A.C et al., 2018), our study demonstrated the abundance of lactobacillus in pregnancy. It also demonstrated the abundance of CST3 and 4 the African race. This is in concurrence with other studies carried out in the African American populations (Anne L.D et al., 2021). However, a cross-sectional study by Anukam K.C et al., 2019 showed CST 1 dominance in healthy Nigerian women. This shows that geographical location also plays a role on type of vaginal microbiome.

Our study did not demonstrate an association between a specific CST and increased risk of preterm. This is in concurrence with findings of David A.M et al., 2015, who in a cross sectional longitudinal multi-ethnic study found no association between a specific CST and the timing of delivery. This finding was similarly found by Freitas A.C et al., 2018. On the contrary, Lindsay M. Kindinger et al.,., 2017 in a cross sectional cohort study established an association between CST 3 and the risk of preterm. They found that L. iners dominance at 16 weeks gestation predicted PTB with 67% sensitivity and 71% specificity. This is similar to findings by Anne L.

Dunlop et al., 2021 who established CST 3 had an intermediate risk of PTB with an odds ratio of 4.1.The study also showed CST 4 had a high risk of PTB with an odd ratio of 7.7.

Our study demonstrated a co-existence between CST 3 and CST 4 in that L. iners (CST 3) was present in 90% of cases while CST 4 was present in 80% of the cases. Similarly, in the controls CST 3 was present in 84% and CST 4 was present in 67%. This compares to a case-control study by Callahan B.J et al., 2017.

As regards to sociodemographic characteristics, the study did not find any significant association between the sociodemographic characteristics and the timing of delivery. This is similar to findings by Peter W. et al., 2018 in a cross-sectional study at KNH that looked at the prevalence and factors associated with PTB at Kenyatta national hospital. On the contrary, systematic reviews by Goldenberg RL et al., 2008 and Muglia LJ et al., 2010 identified several maternal factors that are associated with PTB. This is in contrast to our study; however this is possibly due to four main characteristics of our study, that we excluded patients with known risk factors for PTB such as co-morbidities, multiple pregnancies and cervical incompetence, that all our participants were of the same race, None of the participants were in the extreme ages (<18 and >40) and the primary data questionnaire did not include BMI, history of PTB and substance abuse.

4.2 Conclusions

We confirmed the dominance of CST 3 and 4 in pregnant African women. The study also showed the co-existence of CST 3 and 4, however, there was no association between a specific CST and the risk of preterm. This data implies that preterm birth may be related to other factors other than the vaginal microbiome.

4.3 Recommendations

We recommend longitudinal sampling at different gestational ages up to 6 weeks postpartum. This would enable establishment of the stability of vaginal microbiome throughout pregnancy and changes noted in pueperium.

Study results dissemination plan

I presented the study results to the department of obstetrics and gynecology as part of my fulfillment for conferment of master of medicine. The findings will then be disseminated to Thika level 5 county hospital. I also plan to publish the outcomes for international dissemination.

Study limitations

1 The study was undertaken from bio-banked samples collected earlier for other studies, thus future studies should be prospective.
2 We used 1 cross-sectional sample collected between weeks 20-28weeks, however longitudinal samples collected at grouped gestational ages would be ideal for future studies.
3 Like all metagenomic studies using 16S rRNA gene sequencing, the main limitation is that the technique cannot identify non-bacterial microbes, such as viruses, fungi, or protozoa, all of which have been linked to PTB risk

TIMELINES

	January 2020	December 2020	April 2021	September 2021	October 2021	November 2021	March 2022	may 2022
Concept note								
Completion of proposal								
Proposal presentation								
Ethics approval								
Sample metagenomic analysis and statistical analysis								
Results presentation								
Manuscript writing								
Publishing								

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6. APPENDIX

a. APPENDIX 1: Protocol on Purification of Total DNA from Animal Blood or Cells (DNeasy 96 Protocol extract)

Procedure

1. For blood with non-nucleated erythrocytes, follow step1a; for blood with nucleated erythrocytes, follow step 1b; for cultured cells, follow step 1c.

Blood from mammals contains non-nucleated erythrocytes. Blood from animals, such as birds, fish or frogs, contains nucleated erythrocytes.

1a. Non-nucleated: Pipet 20 µl Proteinase K into each collection microtube. Add 50–100 µl anticoagulated blood per collection microtube. Use a 96-Well-Plate Register (provided) to identify the position of each sample. Adjust the volume to 220 µl each with PBS. Continue with step 2.

Optional: If RNA-free genomic DNA is required, add 4 µl RNase A (100 mg/ml) and incubate for 5 min at room temperature (15–25°C) before continuing with step 2.

Keep the clear covers from the collection microtube racks for use in step 3.

1b. Nucleated: Pipet 20 µl Proteinase K into each collection microtube. Add 5–10 µl anticoagulated blood. Use a 96-Well-Plate Register (provided) to identify the position of each sample. Adjust the volume to 220 µl each with PBS. Continue with step 2.

Optional: If RNA-free genomic DNA is required, add 4 µl RNase A (100 mg/ml) and incubate for 5 min at room temperature before continuing with step 2.

Keep the clear covers from the collection microtube racks for use in step 3.

1c. Cultured cells: Centrifuge the appropriate number of cells (maximum 5 x 106 each) for 5 min at 300 x g. Use a 96-Well-Plate Register (provided) to identify the

position of each sample. Resuspend the pellets in 200 µl PBS each. Add 20 µl Proteinase K each. Continue with step 2.

When using a frozen cell pellet, allow cells to thaw before adding PBS until the pellet can be dislodged by gently flicking the tube.

Ensure that an appropriate number of cells is used in the procedure. For cell lines with a high degree of ploidy (e.g., HeLa cells), it is recommended to use less than the maximum number of cells listed in Table 1 (page 16).

36 DNeasy Blood & Tissue Handbook 07/2020

Optional: If RNA-free genomic DNA is required, add 4 µl RNase A (100 mg/ml). Seal the collection microtubes properly using the caps provided, mix by vortexing, and incubate for 5 min at room temperature before continuing with step 2.

Keep the clear covers from the collection microtube racks for use in step 3.

2. Add 200 µl Buffer AL (without added ethanol).

Ensure that ethanol has not been added to Buffer AL (see “Buffer AL”, page 19).

Buffer AL can be purchased separately (see page 59 for ordering information).

3. Seal the collection microtubes properly using the caps provided.

Place a clear cover (saved from step 1) over each rack of collection microtubes, and shake the racks vigorously up and down for 15 s. To collect any solution from the caps, centrifuge the collection microtubes. Allow the centrifuge to reach 3000 rpm, and then stop the centrifuge.

Do not prolong this step.

Important: The rack of collection microtubes must be vigorously shaken up and down with both hands to obtain a homogeneous lysate. Inverting the rack of collection microtubes is not sufficient for mixing. The genomic DNA will not be sheared by vigorous shaking. The lysate and Buffer AL should be mixed immediately and thoroughly to yield a

homogeneous solution.

Keep the clear covers from the collection microtube racks for use in step 6.

4. Incubate at 56°C for 10 min. Place a weight on top of the caps during the incubation. Mix occasionally during incubation to disperse the sample, or place on a rocking platform.

Note: Do not use a rotary- or vertical-type shaker as continuous rotation may release the caps. If incubation is performed in a water bath make sure that the collection microtubes are not fully submerged and that any remaining water is removed prior to removing the caps in step 5.

5. Carefully remove the caps, and add 200 µl ethanol (96–100%) to each sample.

DNeasy Blood & Tissue Handbook 07/2020 37

6. Seal the collection microtubes properly using the caps provided. Place a clear cover over each rack of collection microtubes, and shake the racks vigorously up and down for 15 s. To collect any solution from the caps, centrifuge the collection microtubes. Allow the centrifuge to reach 3000 rpm, and then stop the centrifuge.

Do not prolong this step.

Important: The rack of collection microtubes must be vigorously shaken up and down with both hands to obtain a homogeneous lysate. Inverting the rack of collection microtubes is not sufficient for mixing. The genomic DNA will not be sheared by vigorous shaking. The lysate and ethanol should be mixed immediately and thoroughly to yield a homogeneous solution.

7. Place two DNeasy 96 plates on top of S-Blocks (provided). Mark the DNeasy 96 plates for later sample identification.

8. Remove and discard the caps from the collection microtubes. Carefully transfer the lysis mixture (maximum 900 µl) of each sample from step 6 to each well of the DNeasy

96 plates.

Take care not to wet the rims of the wells to avoid aerosols during centrifugation. Do not transfer more than 900 µl per well.

Note: Lowering pipette tips to the bottoms of the wells may cause sample overflow and cross-contamination. Therefore, remove one set of caps at a time, and begin drawing up the samples as soon as the pipette tips contact the liquid. Repeat until all the samples have been transferred to the DNeasy 96 plates.

9. Seal each DNeasy 96 plate with an AirPore Tape Sheet (provided). Centrifuge for 4 min at 6000 rpm.

AirPore Tape prevents cross-contamination between samples during centrifugation.

After centrifugation, check that all of the lysate has passed through the membrane in each well of the DNeasy 96 plates. If lysate remains in any of the wells, centrifuge for a further 4 min.

10. Remove the tape. Carefully add 500 µl Buffer AW1 to each sample.

Note: Ensure that ethanol has been added to Buffer AW1 prior to use.

11. Seal each DNeasy 96 plate with a new AirPore Tape Sheet (provided). Centrifuge for 2 min at 6000 rpm.

12. Remove the tape. Carefully add 500 µl Buffer AW2 to each sample.

Note: Ensure that ethanol has been added to Buffer AW2 prior to use.

13. Centrifuge for 15 min at 6000 rpm.

Do not seal the plate with AirPore Tape.

The heat generated during centrifugation ensures evaporation of residual ethanol in the sample (from Buffer AW2) that might otherwise inhibit downstream reactions.

14. Place each DNeasy 96 plate in the correct orientation on a new rack of Elution

Microtubes RS (provided).

15. To elute the DNA, add 200 µl Buffer AE to each sample, and seal the DNeasy 96 plates with new AirPore Tape Sheets (provided). Incubate for 1 min at room temperature. Centrifuge for 4 min at 6000 rpm.

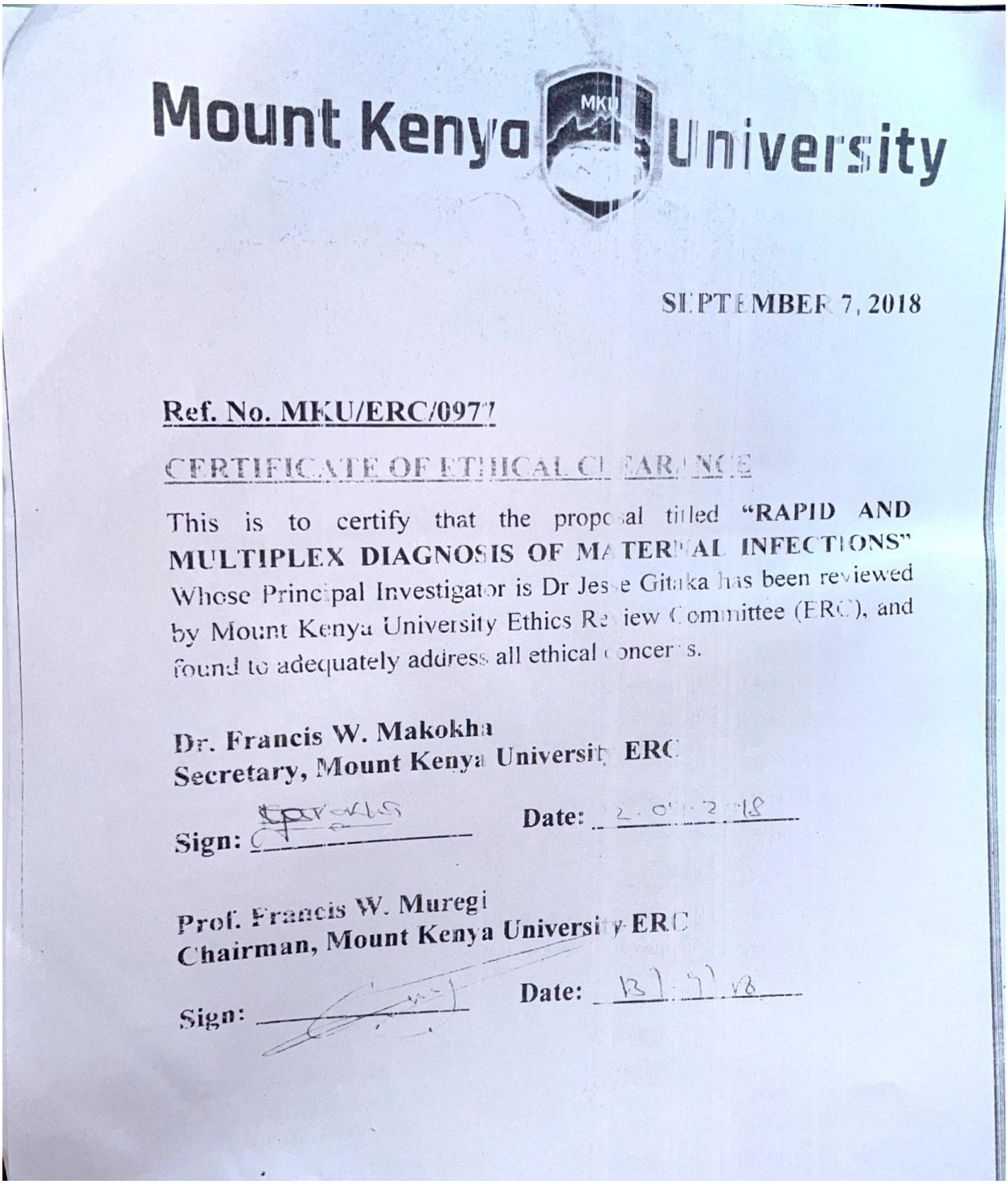
Two hundred microliters Buffer AE is sufficient to elute up to 75% of the DNA from each well of the DNeasy 96 plate.

Elution with volumes less than 200 µl significantly increases the final DNA concentration of the eluate but may reduce overall DNA yield. For samples containing less than 1 µg DNA, elution in 50 µl Buffer AE is recommended.

16. Recommended: For maximum DNA yield, repeat step 15 with another 200 µl Buffer AE. A second elution with 200 µl Buffer AE will increase the total DNA yield by up to 25%. However due to the increased volume, the DNA concentration is reduced. If a higher DNA concentration is desired, the second elution step can be performed using the 200 µl eluate from the first elution. This will increase the yield by up to 15%.

Use new caps (provided) to seal the Elution Microtubes RS for storage.

b. Appendix 2: Ethics approval for the primary study.



Mount Kenya University

SEPTEMBER 7, 2018

Ref. No. MKU/ERC/0977

CERTIFICATE OF ETHICAL CLEARANCE

This is to certify that the proposal titled **"RAPID AND MULTIPLEX DIAGNOSIS OF MATERNAL INFECTIONS"** Whose Principal Investigator is Dr Jesse Gitaka has been reviewed by Mount Kenya University Ethics Review Committee (ERC), and found to adequately address all ethical concerns.

Dr. Francis W. Makokha
Secretary, Mount Kenya University ERC

Sign: _____ **Date:** 12.09.2018

Prof. Francis W. Muregi
Chairman, Mount Kenya University ERC

Sign: _____ **Date:** 13.9.18

c. Appendix 3: clinical information and consent form for the primary study

INFORMED CONSENT FORM (English)

Study no ..... G1 0 .....

Date [illegible]

Title of study

RAPID AND MULTIPLEX DIAGNOSIS OF MATERNAL INFECTIONS

Principal Investigator:

Dr. Jesse Gitaka, MD, PhD (Head of Pure and Applied Sciences Research Programme, Mount Kenya University)

Introduction:

You are being requested to participate in a medical research study on maternal infections. Maternal morbidity and mortality due to infections are particularly high in low income countries like Kenya. These infections are mostly subclinical and contribute significantly to the inflammatory processes that underlie still births and preterm labour and jeopardise the new-born. Rapid and multiplex detection of pathogens that cause the infections during pregnancy can enable prompt treatment and therefore improve pregnancy and neonatal outcomes.

Purpose of study:

This study aims to develop a novel diagnostic tool that will detect multiple bacterial infections simultaneously in pregnant women.

Purpose of consent form:

The purpose of this consent form, therefore, is to give you information that might help you decide whether you would participate in the study or not. Please read the form carefully. You are allowed to ask questions related to the study and implications on your part.

Procedures to be followed:

You will be requested to provide urine sample and a high vaginal swab specimen. You will also be requested to answer questions regarding your health status and that of the baby.

Risks:

You may experience minor discomfort during the collection of vaginal samples. You will only be involved in physical examination, interview, and vaginal and urine sample collection.

Benefits:

The findings of the proposed study will advance the development of rapid tests for diagnosis of maternal infections. This will be useful in channeling the improvement of next generation

diagnostic tools. It will also aid policy makers to guide appropriately on improved diagnostic programs that have significant economic benefits to the society.

Assurance of confidentiality:

A coding system will be used to identify the participants, no self-identifiers such as name will be used. All the information collected is for research purposes only and will be treated with maximal discretion. The records will remain confidential and will not appear when we present this study or publish the results. You will also receive a copy of the consent form.

Handling of specimens:

Immediately after the completion of the experiments, all specimens will be destroyed.

Right to refuse or withdraw:

It is important that you understand the following general principles that will apply to all participants in the study:

1. Participation is entirely voluntary.
2. You may withdraw from this study at any time without penalty or loss of benefits.
3. Please feel free to ask any questions that you may have.

Participant's statement:

I acknowledge that this consent form has been fully explained to me in a language that I understand, and I do agree to participate in the study.

Participant's name..... .....

Participant's Signature..... Date .....

Investigator's signature ..... Date .....

Persons to be contacted in case of any questions:

1. The secretary, MOUNT KENYA UNIVERSITY ETHICAL REVIEW COMMITTEE, P.O. Box 342- 01000 Thika. Tel. 0725809429. Email: research@mku.ac.ke
2. Principal Investigator: Dr. Jesse Gitaka, MD, PhD (Mount Kenya University) P.O. Box 342- 01000 Thika. Tel. 0722425613

d. Appendix 4: clinical data form for the primary study

CLINICAL DATA FORM

Rapid and Multiplex Diagnosis of Maternal Bacterial Infections

Participant Study Number: _____

Study group: _____

MATERNAL PROFILE

Participant Number			
ANC NUMBER	_____		
Study Site (Health Centre Name)	_____		
Inclusion/exclusion criteria *Patient must meet all criteria to eligible for the study	Met all ☐1.	Not met* ☐2.	
Date of Informed Consent	D D M M M Y Y Y Y		
Date of Birth	D D M M M Y Y Y Y	Or estimated age _____	
Gravida			
Parity			
Estimated Gestational Age _____weeks			
Date of Enrolment	D D M M M Y Y Y Y		
Marital status	☐1. S	☐2. M	
Education	☐1.Primary	☐2.Secondary Sch	☐9. University

MEDICAL HISTORY		
Malnutrition_____	Diabetes_____	Preeclampsia_____
HIV_____	Malaria_____	
Family History: Twins Y or N		

Address			
Telephone			
Occupation			
Next of kin			RELATIONSHIP: _____
Next of Kin's contact/phone			

PHYSICAL EXAMINATION (First Visit)

General_____

CVS_____ Resp._____

Breasts_____

Abdomen_____

Vaginal Examination_____

Discharge/GUD_____

Weight in kgs_____ Gestation in weeks_____

Antenatal Profile

Hb_____

Blood Group_____

Rhesus_____

Serology(VDRL/RPR)_____

TB Screening

HIV:

Reactive ☐

Non reactive ☐

Not tested ☐

Urinalysis_____

Bs for Mps_____

Neonatal outcome:

Live
YES

NO

If NO; tick appropriately
Fresh stillbirth _____

Macerated stillbirth _____

APGAR score

Neonatal weight

_____ **grams**

e. Appendix 5: permission to use bio-banked data from principle investigator

Mount Kenya University

TO:
KNH-UoN ERC
Email: uonknh_erc@uonbi.ac

RE: CONSENT TO THE USE OF BIOBANKED PLACENTA SPECIMENS ACQUIRED FOR *"RAPID AND MULTIPLEX DIAGNOSIS OF MATERNAL BACTERIAL INFECTION"* PROJECT (REFERENCE NUMBER: MKU/ERC/0977)

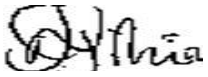
We make reference to the above matter.

I, **Dr. Jesse Gitaka**, the Principal Investigator of the above named study do give my consent
Dr. John Mwangi Kamau to the use of the data and specimens collected in his study titled The vaginal microbiome of women with preterm births versus women with term births who attended ANC at Thika Level 5 County Referral Hospital between January 2019 and March 2019.

Kindly accord him the necessary assistance

Thank you in Advance.

Yours Faithfully;



……
Dr. Jesse Gitaka, MD, MTM, PhD

f. Appendix 6: high vaginal swab collection procedure

1. Explain procedure to woman and gain consent
2. Advise woman to pass urine if needed
3. Ensure privacy is maintained
4. Place woman in lithotomy position
5. Wash hands and put on sterile gloves
6. Lubricate speculum with water-based lubricant
7. Pass speculum gently and locate the cervix
8. Collect specimen from posterior fornix of vagina using a sterile swab stick
9. Open tube containing transport medium, place swab inside immediately, and seal
10. Label HVS with woman's name and study number, the time, and date
11. Place HVS in biohazard plastic bag and place in the cooler box provided. The cooler box shall be collected daily and the samples frozen for future analysis.
12. Rinse reusable speculum and send to Central Sterilizing Department (CSD) for re-sterilizing,
13. or discard single-use speculum in general waste, or contaminated waste bin if heavily contaminated
14. Dispose of other equipment appropriately
15. Remove gloves and wash hands
16. Document in medical record and on antenatal card

g. Appendix 7: ERC consent for this study

UNIVERSITY OF NAIROBI
COLLEGE OF HEALTH SCIENCES
P O BOX 19676 Code 00202
Telegrams: varsity
Tel:(254-020) 2726300 Ext 44355

KNH-UON ERC
Email: uonknh_erc@uonbi.ac.ke
Website: http://www.erc.uonbi.ac.ke
Facebook: https://www.facebook.com/uonknh.erc
Twitter: @UONKNH_ERC https://twitter.com/UONKNH_ERC

KENYATTA NATIONAL HOSPITAL
P O BOX 20723 Code 00202
Tel: 726300-9
Fax: 725272
Telegrams: MEDSUP, Nairobi

Ref: KNH-ERC/A/345

30th September, 2021

Dr. John Mwangi Kamau
Reg. No.H58/11238/2018
Dept. of Obstetrics and Gynaecology
School of Medicine
College of Health Sciences
University of Nairobi

Dear Dr. Kamau

RESEARCH PROPOSAL: DIFFERENCES IN VAGINAL MICROBIOME IN WOMEN WITH PRETERM LABOUR AND THOSE WITH TERM LABOUR THAT ATTENDED ANC AT THIIKA LEVEL 5 COUNTY REFERRAL HOSPITALS BETWEEN JANUARY 2019 AND MARCH 2019 (P398/05/2021)

This is to inform you that the KNH- UoN Ethics & Research Committee (KNH-UoN ERC) has reviewed and **approved** your above research proposal. The approval period is 30th September 2021 – 29th September 2022.

This approval is subject to compliance with the following requirements:

i. Only approved documents (informed consents, study instruments, advertising materials etc) will be used.
ii. All changes (amendments, deviations, violations etc.) are submitted for review and approval by KNH-UoN ERC before implementation.
iii. Death and life threatening problems and serious adverse events (SAEs) or unexpected adverse events whether related or unrelated to the study must be reported to the KNH-UoN ERC within 72 hours of notification.
iv. Any changes, anticipated or otherwise that may increase the risks or affect safety or welfare of study participants and others or affect the integrity of the research must be reported to KNH- UoN ERC within 72 hours.
v. Clearance for export of biological specimens must be obtained from KNH- UoNERC for each batch of shipment.
vi. Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. (Attach a comprehensive progress report to support the renewal).
vii. Submission of an executive summary report within 90 days upon completion of the study.

This information will form part of the data base that will be consulted in future when processing related research studies so as to minimize chances of study duplication and/ or plagiarism.

h. Appendix 8: Addition of Dr. Jesse Gitaka from Mount Kenya University as a supervisor

UNIVERSITY OF NAIROBI
COLLEGE OF HEALTH SCIENCES
SCHOOL OF MEDICINE
Department of Obstetrics & Gynaecology

Kenyatta National Hospital Campus
P.O. Box 19676-00202
NAIROBI
KENYA.
Telephone: 2726300 Ext. 43372
2726300
dept-obsgynaecology@uonbi.ac.ke

REF: H58/11238/2018

March 31, 2021

Dr. Jesse Gitaka
Mount Kenya University

RE: CONFIRMATION AS ADDITIONAL SUPERVISOR FOR DR. JOHN MWANGI KAMAU

The Department has approved your appointment as one of the supervisors of Dr. John Mwangi Kamau for his M. Med Dissertation titled **'Differences in Vaginal Microbiome in Women with Preterm Labour and those with Term Labour that Attended ANC at Thika Level 5 County Referral Hospital Between January 2019 and March 2019'**

Regards

DEPARTMENT OF OBSTETRIC AND GYNAECOLOGY
P.O. Box 19676, NAIROBI
Date 31/3/2021
COLLEGE OF HEALTH SCIENCES
UNIVERSITY OF NAIROBI

PROF. OMONDI OGUTU
CHAIRMAN
DEPARTMENT OF OBSTETRICS AND GYNAECOLOGY