MICROBIAL COLONISATION OF HUMAN FOLLICULAR FLUID AND ADVERSE *IN VITRO* FERTILISATION OUTCOMES

Elise Sarah Pelzer

Bachelor of Applied Science, QUT 2002

Master of Biotechnology, QUT 2006

Institute of Health and Biomedical Innovation

Cell and Molecular Biosciences Discipline

Faculty of Science and Technology

Queensland University of Technology

Brisbane, Australia

A thesis submitted for the degree of Doctor of Philosophy

2011

Precious angels
Born too soon
The challenges of adversity
Gave strength and the drive to persist
The path through the depths of despair
Brought the courage to hope and dream

Celebrate little miracles
Delivered safely
Such an amazing journey
Is life

ABSTRACT

This study, investigating 263 women undergoing trans-vaginal oocyte retrieval for *in vitro* fertilisation (IVF) found that microorganisms colonising follicular fluid contributed to adverse IVF (pre-implantation) and pregnancy (post-implantation) outcomes including poor quality embryos, failed pregnancy and early pregnancy loss (< 37 weeks gestation). Some microorganisms also showed *in vitro* growth patterns in liquid media that appeared to be enhanced by the hormonal stimulation protocol used for oocyte retrieval. Elaborated cytokines within follicular fluid were also associated with adverse IVF outcomes.

This study is imperative because infertility affects 16% of the human population and the numbers of couples needing assistance continues to increase. Despite significant improvements in the technical aspects of assisted reproductive technologies (ART), the live birth rate has not increased proportionally. Overt genital tract infection has been associated with both infertility and adverse pregnancy outcomes (including miscarriage and preterm birth) as a direct result of the infection or the host response to it. Importantly, once inflammation had become established, medical treatment often failed to prevent these significant adverse outcomes. Current evaluations of fertility focus on the ovary as a site of steroid hormone production and ovulation. However, infertility as a result of subclinical colonisation of the ovary has not been reported. Furthermore, identification of the microorganisms present in follicular fluid and the local cytokine profile may provide clinicians with an early indication of the prognosis for IVF treatment in infertile couples, thus allowing antimicrobial treatment and/or counselling about possible IVF failure.

During an IVF cycle, multiple oocytes undergo maturation *in vivo* in response to hormonal hyperstimulation. Oocytes for *in vitro* insemination are collected trans-vaginally. The follicular fluid that bathes the maturing oocyte *in vivo*, usually is discarded as part of the IVF procedure, but provides a unique opportunity to investigate microbial causes of adverse IVF outcomes. Some previous studies have identified follicular fluid markers that predict IVF pregnancy outcomes. However, there have not been any detailed microbiological studies of follicular fluid.

For this current study, paired follicular fluid and vaginal secretion samples were collected from women undergoing IVF cycles to determine whether microorganisms in follicular fluid were associated with adverse IVF outcomes. Microorganisms in follicular fluid were regarded as either "colonisers" or "contaminants"; colonisers, if they were unique to the follicular fluid sample, and contaminants if the same microorganisms were detected in the vaginal and follicular fluid samples indicating that the follicular fluid was merely contaminated during the oocyte retrieval process. Quite unexpectedly, by these criteria, we found that follicular fluid from approximately 30% of all subjects was colonised with bacteria. Fertile and infertile women with colonised follicular fluid had decreased embryo transfer rates and decreased pregnancy rates compared to women with contaminated follicular fluids. The observation that follicular fluid was not always sterile, but contained a diverse range of microorganisms, is novel. Many of the microorganisms we detected in follicular fluid are known opportunistic pathogens that have been detected in upper genital tract infections and are associated with adverse pregnancy outcomes.

Bacteria were able to survive for at least 28 weeks *in vitro*, in cultures of follicular fluid. Within 10 days of establishing these *in vitro* cultures, several species (*Lactobacillus* spp., *Bifidobacterium* spp., *Propionibacterium* spp., *Streptococcu*s spp. and *Salmonella entericus*) had formed biofilms. Biofilms play a major role in microbial pathogenicity and persistence. The propensity of microbial species to form biofilms in follicular fluid suggests that successful treatment of these infections with antimicrobials may be difficult.

Bifidobacterium spp. grew, in liquid media, only if concentrations of oestradiol and progesterone were similar to those achieved *in vivo* during an IVF cycle. In contrast, the growth of *Streptococcus agalactiae* and *Escherichia coli* was inhibited or abolished by the addition of these hormones to culture medium. These data suggest that the likelihood of microorganisms colonising follicular fluid and the species of bacteria involved is influenced by the stage of the menstrual cycle and, in the case of IVF, the nature and dose of steroid hormones administered for the maturation of multiple oocytes *in vivo*. Our findings indicate that the elevated levels of steroid hormones during an IVF cycle may influence the microbial growth within follicular fluid, suggesting that the treatment itself will impact on the microflora present in the female upper genital tract during pre-conception and early post-conception phases of the cycle.

The effect of the host immune response on colonising bacteria and on the outcomes of IVF also was investigated. White blood cells reportedly compose between 5% and 15% of the cell population in follicular fluid. The follicular membrane is semi-permeable and cells are actively recruited as part of the normal menstrual cycle and

in response to microorganisms. A previous study investigated follicular fluid cytokines from infertile women and fertile oocyte donors undergoing IVF, and concluded that there were no significant differences in the cytokine concentrations between the two groups. However, other studies have reported differences in the follicular fluid cytokine levels associated with infertile women with endometriosis or polycystic ovary syndrome. In this study, elevated levels of interleukin (IL)-1 α, IL-1 β and vascular endothelial growth factor (VEGF) in vaginal fluid were associated with successful fertilisation, which may be useful marker for successful fertilisation outcomes for women trying to conceive naturally or prior to oocyte retrieval for IVF. Elevated levels of IL-6, IL-12p40, granulocyte colony stimulating factor (GCSF) and interferon-gamma (IFN γ) in follicular fluid were associated with successful embryo transfer.

Elevated levels of, pro-inflammatory, IL-18 and decreased levels of, anti-inflammatory, IL-10 were identified in follicular fluid from women with idiopathic infertility. Successful fertilisation and implantation is dependent on a controlled pro-inflammatory environment, involving active recruitment of pro-inflammatory mediators to the genital tract as part of the menstrual cycle and early pregnancy. However, ongoing pregnancy requires an enhanced anti-inflammatory environment to ensure that the maternal immune system does not reject the semi-allergenic foetus. The pro-inflammatory skew in the follicular fluid of women with idiopathic infertility, correlates with normal rates of fertilisation, embryo discard and embryo transfer, observed for this cohort, which were similar to the outcomes observed for fertile women. However, their pregnancy rate was reduced compared to fertile

women. An altered local immune response in follicular fluid may provide a means of explaining infertility in this cohort, previously defined as 'idiopathic'.

This study has found that microorganisms colonising follicular fluid may have contributed to adverse IVF and pregnancy outcomes. Follicular fluid bathes the cumulus oocyte complex during the *in vivo* maturation process, and microorganisms in the fluid, their metabolic products or the local immune response to these microorganisms may result in damage to the oocytes, degradation of the cumulus or contamination of the IVF culture system. Previous studies that have discounted bacterial contamination of follicular fluid as a cause of adverse IVF outcomes failed to distinguish between bacteria that were introduced into the follicular fluid at the time of trans-vaginal oocyte retrieval and those that colonised the follicular fluid. Those bacteria that had colonised the fluid may have had time to form biofilms and to elicit a local immune response. Failure to draw this distinction has previously prevented consideration of bacterial colonisation of follicular fluid as a cause of adverse IVF outcomes.

Several observations arising from this study are of significance to IVF programs. Follicular fluid is not always sterile and colonisation of follicular fluid is a cause of adverse IVF and pregnancy outcomes. Hormonal stimulation associated with IVF may influence whether follicular fluid is colonised and enhance the growth of specific species of bacteria within follicular fluid. Bacteria in follicular fluid may form biofilms and literature has reported that this may influence their susceptibility to antibiotics. Monitoring the levels of selected cytokines within vaginal secretions may inform fertilisation outcomes.

This study has identified novel factors contributing to adverse IVF outcomes and that are most likely to affect also natural conception outcomes. Early intervention, possibly using antimicrobial or immunological therapies may reduce the need for ART and improve reproductive health outcomes for all women.

LIST OF KEYWORDS

Assisted reproductive technology (ART); follicular fluid; upper genital tract; lower genital tract; steroid hormones; oestradiol; progesterone; biofilm; *Lactobacillus* species; trans-vaginal oocyte retrieval; semen; washed semen; cytokines; microorganisms; endometriosis; polycystic ovary syndrome; genital tract infection; genital tract colonisation or contamination; male factor infertility; fertilisation; embryo transfer; pregnancy, *in vitro* fertilisation (IVF).

LIST OF PUBLICATIONS AND MANUSCRIPTS

The following publications and manuscripts have been prepared in conjunction with this thesis.

Pelzer, E. S., Allan, J. A., Cunningham, K., Mengersen, K., Allan, J. M., Launchbury, T., Beagley, K. and Knox, C. L. (2011) Microbial colonisation of follicular fluid: alterations in cytokine expression and adverse assisted reproductive technology outcomes. Hum Reprod. 26 (7):1799-1812.

The candidate contributed 80% of the work for this manuscript, which included primary culture analysis of follicular fluid and vaginal secretion microflora, performance and interpretation of cytokine assays, statistical analysis and drafting and approval of the final manuscript. This work is presented in Chapter six of this thesis.

Pelzer, E. S. (2010) 'Sexually Transmitted Infections and Infertility', film on the Sexual and Reproductive Health and Blood-borne Virus Resources CD, Family Planning Queensland and Queensland Health, Version 5.

ABSTRACTS AND PRESENTATIONS

Pelzer, E. S., Cunningham, K., Allan, J. A., Mengersen, K., Allan, J. M., Launchbury, T., Beagley, K. and Knox, C. L. Microbial colonisation of follicular fluid: alterations in cytokine expression and adverse assisted reproductive technology outcomes. *IHBI Inspires Postgraduate Conference, Gold Coast* (2010 – Best oral presentation)

Pelzer, E. S., Allan, J. A., Allan, J. M., Launchbury, T., and Knox, C. L. *In vitro* characterisation of biofilm formation in follicular fluid *Society for Reproductive Biology Annual Conference, Sydney* (2010 – New Investigator Award Finalist, Oral presentation)

Pelzer, E. S., Cunningham, K., Allan, J. A., Mengersen, K., Allan, J. M., Launchbury, T., Beagley, K. and Knox, C. L. Microbial colonisation of follicular fluid: alterations in cytokine expression and adverse assisted reproductive technology outcomes. *International Congress of Reproductive Immunology Annual conference, Cairns* (2010 – Award finalist, oral presentation)

Pelzer, E. S., Allan, J. A., Allan, J. M., Launchbury, T., and Knox, C. L. Microbial colonisation of follicular fluid. Scientists *in Reproductive Technology Annual Conference, Gold Coast* (2010 – invited speaker presentation)

Pelzer, E. S., Cunningham, K., Allan, J. A., Mengersen, K., Allan, J. M., Launchbury, T., Beagley, K. and Knox, C. L. Microbial colonisation of follicular fluid: alterations in cytokine expression and adverse assisted reproductive technology outcomes. *ASMR Postgraduate Student Conference, Brisbane* (2010 – Finalist poster presentation)

Pelzer, E. S., Allan, J. A., Allan, J. M., Launchbury, T. and Knox, C. L. Colonisation of follicular fluid; the effect on assisted reproductive technology outcomes. *Australian Society of Microbiology Annual Scientific Meeting, Melbourne* (July 2009 – oral presentation).

Pelzer, E. S., Allan, J. A., Allan, J. M., Launchbury, T. and Knox, C. L. Colonisation of follicular fluid; the effect on assisted reproductive technology outcomes. *Fertility Society of Australia Annual Conference, Brisbane* (October 2009 – electronic poster presentation)

Pelzer, E. S., Allan, J. A., Allan, J. M., Launchbury, T., Bosci, D. and Knox, C. L. Non-sterile follicular fluid: a reason for adverse assisted reproductive technology outcomes? *ASMR Postgraduate Student Conference, Brisbane* (2009 – poster presentation)

Pelzer, E. S., Allan, J. A., Allan, J. M., Launchbury, T. and Knox, C. L. Biofilm formation in human follicular fluid: friend or foe? *IHBI Inspires Postgraduate Conference, Brisbane* (2009 – oral presentation)

Pelzer, E. S., Allan, J. A., Allan, J. M., Launchbury, T., Bosci, D. and Knox, C. L. Colonisation of follicular fluid: a reason for adverse assisted reproductive technology and pregnancy outcomes. *Infection and Immunity Conference, Gold Coast* (2009 – poster presentation)

Pelzer, E. S., Allan, J. A., Allan, J. M., Launchbury, T., Bosci, D. and Knox, C. L. Colonisation of follicular fluid; the effect on assisted reproductive technology outcomes. *Public Health Association of Australia Conference, Brisbane* (2008 – oral presentation)

Pelzer, E. S., Allan, J. A., Allan, J. M., Launchbury, T., Bosci, D. and Knox, C. L. Colonisation of follicular fluid; the effect on assisted reproductive technology outcomes. *IHBI Inspires Postgraduate Conference, Gold Coast* (2008 – Runner-up oral presentation)

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

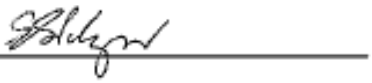
ART	Assisted reproductive technology
CART	Classification and regression tree
CC, CXC, C, CX_3C	Chemokine
CCR, CCXR	Chemokine receptor
cDNA	Complementary DNA
CDS	Calibrated dichotomous sensitivity
CFU	Colony forming unit
CMV	Cytomegalovirus
CoNS	Coagulase-negative Staphylococci
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EBV	Epstein barr virus
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
FSH	Follicle stimulating hormone
GCSF	Granulocyte colony stimulating factor
GMCSF	Granulocyte macrophage colony stimulating factor
Gro	Growth regulated oncogene
H_2O_2	Hydrogen peroxide
HBV	Hepatitis B virus
hCG	Human chorionic gonadotropin
HCV	Hepatitis C virus
HIV	Human Immunodeficiency Virus

HPV	Human papilloma virus
HRM	High-resolution melt
HSV	Herpes simplex virus
HTLV	Human T cell leukaemia virus
ICSI	Intra-cytoplasmic sperm injection
IFN	Interferon
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
IL	Interleukin
IVF	*In vitro* fertilisation
LGT	Lower genital tract
LH	Luteinising hromone
LIF	Leukaemia inhibitory factor
LTR	Long terminal repeat
M	Molar
MCSF	Macrophage colony stimulating factor
MHC	Major histocompatibility complex
MIP	Macrophage inflammatroy protein
mRNA	Messenger ribonucleic acid
ND	Not detected
NK	Natural killer
NS	Not significant
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PDGF	Platelet-derived growth factor
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase PCR
SCF	Sertoli cell factor
sICAM	Secretory intercellular adhesion molecule
STI	Sexually transmitted infection
TBE	Tris borate EDTA
T_h	T helper cell
T_m	Melting temperature
TNF	Tumour necrosis factor
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
UGT	Upper genital tract
VEGF	Vascular endothelial growth factor

STATEMENT OF ORIGINAL AUTHORSHIP

The work presented in this thesis has not been previously submitted to meet requirements for an award at this or any other higher education institution. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person except where due reference is made.

Signed: 

Elise Sarah Pelzer B. App. Sci. (QUT) M. Biotech. (QUT)

Date: 18/10/2011

ACKNOWLEDGEMENTS

I gratefully acknowledge my supervisors, Dr Christine Knox, Associate Professor John Aaskov and Professor Ken Beagley for their encouragement and guidance during my PhD project. I feel very fortunate that my persistence convinced Christine to take me on as a student, which allowed me to embark on a research career in reproductive health, a field about which I am very passionate. Thanks also to my collaborators at Wesley Monash IVF: Drs John and Janet Allan, IVF clinicians, IVF scientists and IVF nurse co-ordinators. I wish to thank the Wesley Monash IVF Service: consulting IVF specialists Doctors John Chenoweth, Stephen Baines, Stephen Cook, Ross Turner and Dana Moisuc who collected follicular fluid specimens and vaginal swabs. My thanks also to the IVF scientists Marta Gutierrez, Tara Launchbury and Pan Ouk who aliquotted follicular fluid and semen samples; and the IVF nurse coordinators Cheryl Morton, Angela Banning, Karen Dever, Kara Carter and Tanya Wallace for enrolling patients in this study and assisting with retrospective collation of ART outcome data. I am also most grateful to Audrey Partenan, Jann Heynes and April Peteru-Barnard for transferring specimens and storing them at the Wesley Research Institute Tissue Bank.

Thanks goes to QUT, IHBI, and the Wesley Research Institute for allowing me to undertake this project and for providing my scholarship, travel and research funding. Professor Ross Young has demonstrated and unrivalled enthusiasm and appreciation for scientific research, and I thank him for his ongoing support of my work. I'd also like to acknowledge the support of the other members in my research group (Professor Kerrie Mengersen, Associate Professor Louise Hafner, Associate Professor Flavia Huygens, Dr Kelly Cunningham, Dr Candice Mitchell, Ashkan

Amirshahi, Samantha Dando and Professor Peter Timms) for their scientific advice and for providing the laughs, particularly during the writing-up process. I wish to acknowledge Jess Harris for keeping me company in the new lab at the WRI, for the challenges of 72-hour experiments with furry friends and for keeping me up to date with modern day lingo. My thanks also to Dr Mary Waterhouse at the Wesley Research Institute for her help with my never-ending statistical questions. I am extremely grateful to Dr Mark O'Brien who openly shared his passion for microbiology with me and provided the foundation on which my love of bugs is based. I also extend a big thank-you to my mentor, Professor Julie Campbell for her 'non-PC' advice and critical appraisal of my thesis.

I would like to thank my family – Steve, my beautiful girls, Rachael, Neve and Stella, Mum and Dad, Nat, Michale, Nicholas, Phillip and Lachlan for their continued support and understanding over the past three-and-a-half years, and for encouraging my pursuits and trying to understand what I was on about.

A special thank-you goes to Steve, who was by my side throughout this entire journey and who provided endless support, love and encouragement, especially when the going got tough and life through some spanners, making the climb seem impossible. Without his support, this journey may not have eventuated or been completed.

I am also grateful to the reviewers of this thesis, for their valuable time, comments and recommendations.

PUBLISHED ARTICLE

Chapter Six has been published and is included in this manuscript as a traditional thesis chapter. Remaining chapters are being prepared for submission to journals.

CITATION STYLE

The citation style used in this thesis is that of the reproductive health journal Human Reproduction. References are listed in alphabetical order of the family name of the first author. In text citations are represented by the family name of the first author and the year of publication for more than two authors and for two authors, the family name and the year of publication for each of the two authors appears in text. The thesis is presented in United Kingdom English.

CHAPTER ONE

INTRODUCTION

1.1 A description of the scientific problem investigated

Infertility is a global problem affecting approximately 16% of couples (Zegers-Hochschild *et al.*, 2009). The total fertility rate continues to decline, and is now down to 1.6 children per woman in developed countries, which is considered insufficient for replacement of the current population (World Health Organisation, 2010). In late 2009, the World Health Organisation in conjunction with the International Committee for Monitoring Reproductive Technologies, for the first time, listed infertility as a disease in the 'International Glossary of ART terminology' (Zegers-Hochschild *et al.*, 2009). This decision acknowledged not only the social, financial and emotional burden such a diagnosis entails, but highlights the need for ongoing research to improve treatment for infertile couples. Idiopathic infertility, infertility with no known cause, affects up to 20% of infertile couples (Wang *et al*., 2009). Investigations into the presence of microorganisms within the upper genital tract may identify further causes for infertility in this population.

Research within the field of ART has resulted in refined technical procedures for processes involved in gamete manipulation (Palermo *et al.*, 1992), genetic screening of embryos (Munne *et al.*, 1993), storage of gametes, embryos and ovarian tissue (Mukaida *et al.*, 2003), and the transfer of gametes or embryos to the female upper genital tract (Gardner *et al.*, 1998, Geary and Moon, 2006). Clinical research has focussed on understanding the mechanisms (genetic, endocrine, immunological) associated with various aetiologies of infertility and the adverse ART outcomes linked to each of these factors. An area of research that has not yet been fully investigated is the effect that microorganisms colonising the female upper genital tract have on ART treatment, success rates and pregnancy outcomes.

Sexually transmitted infections of males and females remains the main preventable cause of global infertility (World Health Organisation, 2006). However, in the absence of clinical symptoms or a diagnosis of sexually transmitted infection, the impact of microorganisms within the genital tract receives little attention. The role of microbial infection in adverse pregnancy outcomes, including miscarriage, preterm rupture of placental membranes, preterm labour and preterm birth in natural conceptions and ART pregnancies is well established (Hillier *et al.*, 1988, Eckert *et al.*, 2003, Goldenberg *et al.*, 2000, Romero *et al.*, 2003). Recent studies have reported evidence of *in vivo* colonisation of the placental tissues in 25 – 79% of placentas (Onderdonk *et al.*, 2008 a, b) and in approximately 80% of endometrial specimens (Andrews *et al.*, 2005) of women who deliver preterm. Cultures of lower genital tract specimens have proved to be poor indicators of the presence of microorganisms within the upper genital tract and discordant results for microbial species were obtained when sampling both the upper and lower genital tract sites (Cicinelli *et al.*, 2009, Spence *et al.*, 1982). A previous study detected bacteria within follicular fluid collected at the time of trans-vaginal oocyte retrieval; however, these bacteria were not associated with adverse ART outcomes when the IVF culture system as a whole was assessed (Cottell *et al.*, 1996). Antimicrobial properties of follicular fluid have also been demonstrated (Gurgan *et al.*, 1993, Stepanovic *et al.*, 2003). A fundamental step in our understanding of the impact of asymptomatic colonisation of the ovarian follicles on ART outcomes will be the characterisation of microorganisms present at this anatomical site.

Microorganisms secrete metabolites into their environment and these prior to fertilisation may damage the maturing oocyte, which is bathed in follicular fluid.

Microorganisms can also elicit an immune response from the host and these responses may affect the oocyte and result in poor quality embryos. To date, no studies have investigated the effect of bacteria colonising follicular fluid on ART outcomes and the immune response in women undergoing ART cycles. Nor has the prevalence of microorganisms and their ability to survive within follicular fluid been previously demonstrated or characterised.

1.2 The overall objectives and hypotheses of the study

The overall objective of this study was to test follicular fluid for the presence of microorganisms and to: identify and characterise the microorganisms present at the time of trans-vaginal oocyte retrieval; determine whether the presence of microorganisms represented colonisation, or was due to contamination of the follicular fluid at the time of oocyte retrieval; determine if their presence modulated the immune response or the secretion of cytokines; and determine whether the presence of microorganisms and/or an immune response to these microorganisms resulted in adverse IVF and pregnancy outcomes[1].

The methods utilised in this study were designed to ensure that true colonisation of follicular fluid may be distinguished from contamination of follicular fluid (as a result of trans-vaginal oocyte retrieval). Vaginal secretions were processed in parallel so that the culture and cytokine assay results for follicular fluid and vaginal secretions may be compared directly.

The central hypothesis of this study is that follicular fluid is colonised by microorganisms and that these microorganisms, or the host immune response to their

[1]This study adopted the definitions for ART and adverse pregnancy outcomes as defined in the Revised Glossary of ART Terminology, Zegers-Hochschild *et al.*, 2009)

presence, adversely affects ART pregnancy outcomes. Thus the study hypothesis is that *colonisation of follicular fluid may be another cause of infertility*. The hypothesis was explored in different cohorts of infertile couples undergoing ART treatment who were classified according to the aetiology of infertility: (1) women with endometriosis, (2) women with polycystic ovary syndrome, (3) women with a history of upper genital tract infection, (4) fertile women (from couples with infertile male partners – the control cohort) and (5) women with idiopathic infertility. Within each cohort, women were classified as having either colonised follicular fluid (microorganisms not also present in the paired vaginal swab, so these microorganisms were present as coloniser(s) prior to oocyte retrieval), or contaminated follicular fluid (microorganisms present in the follicular fluid were also present in the vaginal swab cultures and these microorganisms were introduced into the follicular fluid at the time of oocyte retrieval) and were subsequently compared for each cohort classification and IVF pregnancy outcomes.

The growth characteristics of microorganisms within follicular fluid were further investigated using *in vitro* assays to determine if the bacteria within follicular fluid may form biofilms. The majority of follicular fluids demonstrated polymicrobial colonisation, but did not appear visibly infected/turbid despite the presence of high numbers of colony forming units. The assumption of this study is that the microorganisms may be growing as a biofilm lining the pre-ovulatory follicle, which is biopsied at the time of oocyte retrieval.

The cytokine profile (immune response) to microorganisms within both the follicular fluid and vaginal secretions was also examined to determine whether there was site-

dependent variation of cytokine expression, and whether these cytokines may be correlated with IVF pregnancy outcomes.

1.3 The specific aims of the study

The specific aims of this study were to:

1. determine the prevalence and identity of microorganisms within follicular fluid collected at the time of trans-vaginal oocyte retrieval
2. determine whether these microorganisms represent colonisation of the ovarian follicle or contamination from the vagina at the time of oocyte retrieval
3. compare IVF pregnancy outcomes for women with colonised versus contaminated follicular fluid.
4. determine whether microorganisms within follicular fluid and vaginal secretions (colonised prior to oocyte retrieval or contaminated at the time of oocyte retrieval) generate discriminatory cytokine profiles capable of predicting IVF outcomes or IVF pregnancy outcomes.
5. characterise the growth of bacteria within follicular fluid *in vitro*.
6. investigate the ability of bacterial species prevalent within follicular fluid to grow in the presence of high concentrations of the steroid hormones oestradiol and progesterone (at concentrations comparable to those found in hyperstimulated women).
7. determine the ability of colonising species to persist over time within a biofilm.

CHAPTER TWO

LITERATURE REVIEW

Overview

Despite extensive research and advancements in ART procedures, *in vitro* fertilisation (IVF) live birth rates per treatment cycle have increased by only 1.1% in the past five years. The most recent Australasian statistics (for 2008) from the National Perinatal Statistics Unit (Wang *et al*., 2010) indicate that of 58740 autologous IVF treatment cycles commenced (fresh and frozen embryo transfers), 91.5% of the fresh cycles resulted in oocyte retrieval, 84.5% in fertilisation *in vitro* and embryo transfer, resulting in a clinical pregnancy rate of 22.7% and a live birth rate of 17.2% per treatment cycle commenced. These outcomes highlight the fact that not all IVF treatment cycles result in successful oocyte retrieval, fertilisation or embryo development and therefore, the return of embryos to the female partner does not occur for all couples (Table 2.1). By comparison, ten years previously, 8172 fully stimulated IVF treatment cycles resulted in 896 live births, yielding a live birth rate of 13.3% per treatment cycle commenced. Clearly, the number of ART cycles has improved dramatically in the last decade; however, we believe that further improvements are still possible. This review will focus on infectious aetiologies of upper genital tract (UGT) infections (not limited to sexually transmitted infection) in female and male partners, an insufficiently investigated area of research within the field of ART. A greater understanding of the microorganisms present in the upper genital tract and their role in IVF outcomes may lead to informed therapeutic treatment options and improved ART pregnancy and live birth outcomes.

Table 2.1 Assisted reproduction outcomes, Australia 2004 and 2008

Year	Commenced cycles (autologous and donor)	Oocyte retrieval Rate[1] (%)	Embryo transfer Rate[1] (%)	Clinical pregnancy Rate[1] (%)	Miscarriage Rate[1] (%)	Preterm birth Rate[1] (%)	Live birth Rate[1] (%)
2004	41904	90.8	81.6	21.0	20.1	20.6	16.2
2008	58740	91.5	84.5	22.7	20.6	19.3	17.2

(Wang *et al*., 2010) [1] of total cycles commenced

Globally, similar trends are observed in the field of ART (Table 2.2). The five major regions (Asia, Australia/New Zealand, Europe, Latin America, Middle East and North America) have also reported increased IVF cycle numbers and clinical pregnancy rates, as well as a slight rise in the live birth rates, but these improvements remain low, leaving substantial room for improvement. Preterm birth rates remain high and from 2000 – 2002 there was an 8.1% increase in the miscarriage rate in IVF pregnancies in Australia and New Zealand (Wang *et al*., 2010).

Table 2.2 Global ART statistics 2000 and 2002 (most complete recent data available)

Year	Region	Cycles	Oocyte retrieval	Embryo transfer	Clinical pregnancy Rate	Multiplicity (%)	Miscarriage Rate (%)	Preterm delivery Rate (%)	Live birth Rate
2000	Asia	29957[1]	29957		23.0	27.1	NA	NA	13.8
2002	Asia	115713	44909	7539	26.8	23.1	26.0	21.5	20.0
2000	Australia NZ	16981[1]	16981	24915	21.8	24.9	10.2	32.6	17.1
2002	Australia NZ	33698	17816	27154	26.5	21.9	18.3	27.3	20.4
2000	Europe	226937	207004	98215	25.6		24.2		17.5
2002	Europe	444049	241107		26.7	24.5	20.5	NA	17.9
2000	Latin America	13513	12286	3249	25.2	32.3	NA	NA	19.8
2002	Latin America	24742	14155		30.8	31.7	20.7	28.6	24.4
2000	Middle East	49644	33249	17774	25.5	36.8	NA	NA	17.5
2002	Middle East	69670	29345		28.3	27.0	36.8	35.0	21.8
2000	North America	79463	68254	28687	36.1	35.3	NA	NA	29.3
2002	North America	132413	79894		39.9	34.4	17.7	32.2	32.5

(Zegers-Hochschild *et al*., 2009)

2.1 Introduction

Reproductive failure is defined as: (1) failure to conceive after 12 months of unprotected sexual intercourse (*i.e.* infertility); (2) repeated implantation failure

following ART cycles; or (3) recurrent miscarriage without difficulty of conceiving (natural conceptions). For many couples who are unable to conceive naturally, ART provides a vital last-resort, thus the quality and the successful outcome of ART treatment is paramount. Not only is this process complex and costly, but also an extremely difficult emotional undertaking for the couple involved. IVF treatment cycles are expensive: in 2009, the cost of a single fully stimulated IVF treatment cycle was $6400. The cost is even higher for international, full fee-paying couples who in the same year paid a baseline rate of $15600, excluding travel, accommodation or hospitalisation expenses in the event of complications. These fees do not include additional services, such as intracytoplasmic spermatozoa injection (ICSI), blastocyst culture, assisted embryo hatching, embryo glue or pre-implantation genetic testing (Wesley-Monash IVF personal communication).

The IVF process is complex and often requires numerous treatment cycles. Each IVF treatment cycle begins with the female partner receiving hormonal stimulation to produce multiple mature oocytes, which upon maturation are retrieved by trans-vaginal oocyte retrieval (Wikland *et al.*, 1987). The oocytes are inseminated by IVF or ICSI and then transferred to the uterus at the 2, 4, 8-cell, or the blastocyst stage (Gardner *et al.*, 1998). In the past decade, ART outcomes have improved, mainly due to the improvements in ART techniques and formulations of culture media (Geary and Moon, 2006). In addition, the development of micromanipulation, which permits the scientist to inject a single spermatozoon into an oocyte, improved ART outcomes for couples with male factor infertility (Palermo *et al.*, 1992). Furthermore, the development of sequential media for embryo culture *in vitro* now allows embryos to be cultured to the later blastocyst stage, enabling the assessment of the

developmental potential of the embryos prior to embryo transfer, thus improving the chances of successful outcomes (Gardner *et al.*, 1998). Finally, further reasons for ART failures can be now screened for by collecting single embryonic cells by biopsy and testing for chromosomal abnormalities. If chromosomal abnormalities are detected in embryos, such as aneuploidy, single gene disorders or translocations, then the embryos are discarded. Preimplantation genetic diagnostics routinely rely on fluorescence *in situ* hybridisation for detection of abnormalities in chromosomes X, Y, 13, 18 and 21 by specific probes. If these first five probes return normal results, then chromosomes 16 and 22 are subsequently tested (Munne *et al.*, 1993). Polymerase chain reaction (PCR) is used to detect single-gene disorders (Handyside *et al.*, 1992) including cystic fibrosis, α 1 antitrypsin deficiency, retinitis pigmentosa, haemophilias A and B, thalassemia, Gancher's, Tay Sach's and sickle cell disease. Despite all these preventive measures taken, the preterm birth and miscarriage rates also remain high.

Prior to entry into ART cycles, couples are screened (and treated) for the presence of STIs, including *Chlamydia trachomatis*, gonorrhoea and the blood-borne viral diseases hepatitis; human immunodeficiency virus (HIV) and cytomegalovirus (CMV) (Zegers-Hochschild *et al.*, 2009). However, routine microbiological screening of specimens is not performed for each ART treatment cycle.

This literature review will report on and discuss previous studies of genital tract infections in males and females. Initially, the review will describe the normal flora of the female and male reproductive tracts and discuss the mechanisms of opportunism and ascending invasive infections, whereby microorganisms can gain access to the

UGT. Major microbial pathogens (including those causing sexually transmitted infections) − bacteria, fungi, parasites and viruses − known to infect the lower genital tract (LGT) and/or UGT will be characterised and compared. The reported incidences of LGT and UGT infections and the recognised effects of these on IVF outcomes will be reviewed and possible implications of these findings for improving IVF treatment outcomes will be discussed.

To date, there have been few investigations of the relationship between genital tract microorganisms belonging to the normal regional flora of the genital tract and adverse ART outcomes. However, relationships between adverse pregnancy outcomes and infection have been reported, suggesting that asymptomatic microbial colonisation of the genital tracts of males and females may have a role in adverse IVF outcomes.

2.2 The normal flora of the genital tract and opportunistic pathogens

In the female body, the LGT is comprised of the urethra, vagina and cervix and is inhabited by a rich normal regional flora. Typical infections of the female LGT can result in urethritis, vulvitis, vaginitis and cervicitis (Holmes *et al*., 2008). In the males, the penis and urethra form the LGT, where a rich normal regional flora resides (Wilson, 2005). Infections of the male LGT include balanitis, orchitis and urethritis (Martin, 2008). The female UGT comprises the endometrium, uterine tubes and ovaries, whilst in males the UGT comprises the prostate gland and epididymis. The UGT in both females and males is reported to be a 'normally sterile' site (Holmes *et al*., 2008, Wilson, 2005). However, members of the normal regional flora, microbial pathogens and STIs can cause ascending invasive infections,

including endometritis, pelvic inflammatory disease, salpingitis, oophoritis, tubo-ovarian abscesses and peritonitis (Paavonen *et al*., 2008). Infections in the male UGT result in epididymitis and prostatitis (Geisler *et al*., 2008, Krieger, 2008). The sequelae of these UGT infections can include tissue damage, subsequent infertility and adverse pregnancy outcomes.

Maintaining the optimal balance of urogenital tract microorganisms is important, as the normal regional flora of the female LGT is the first line of defence against potential pathogens. This flora consists predominantly of *Lactobacillus* spp., but may also include *Enterococci*, *Corynebacterium* species, *Gardnerella vaginalis*, *Bacteroides* spp., *Ureaplasma* spp. and *Candida albicans.* Adverse pregnancy outcomes can occur as a result of opportunistic infection, whereby one or more members of the normal regional flora of the urogenital tract overgrow (Onderdonk *et al.*, 2008 a, b). Microorganisms that gain access to the 'normally sterile' UGT can cause infertility (Swenson and Schachter, 1984), decreased fertilisation and conception rates and adverse pregnancy outcomes including miscarriage, intra-amniotic infection, premature rupture of membranes, preterm birth and maternal morbidity, as well as neonatal morbidity or even mortality (Holmes *et al*., 2008). During the ART procedure, the normal defence systems of the female reproductive tract are bypassed or compromised by hormonal hyperstimulation, potentially facilitating microbial pathogenesis. Alterations in the concentration of the ovarian steroid hormones, oestradiol and progesterone, throughout the menstrual cycle, have been associated with changes in susceptibility to genital tract infections (Sonnex, 1998). In addition, the oocyte retrieval, embryo transfer to the uterus and *in vitro* insemination procedures are potential routes by which microorganisms can gain

access to the genital tract or to the oocyte (Fanchin *et al.*, 1998, Tureck, *et al* 1993, Vigil *et al.*, 2002 a, b)

2.3 Female reproductive tract microbiology

2.3.1 The LGT and lactobacillus dominant flora

Lactobacillus spp. account for 90–95% of the normal vaginal flora in most women (Hillier *et al.*, 1990). Lactobacillus produces H_2O_2 and lactic acid, inhibiting the growth of many potential pathogens due to a lowered vaginal pH of <4.5, and the lethal combination of H_2O_2, halide ions and peroxidase (Klebanoff and Coombs, 1991, Klebanoff *et al.*, 1991, Martin *et al.*, 1999). At puberty, as oestrogen levels increase, the vaginal pH decreases and *Lactobacillus* spp. dominate the vaginal microflora (Brabin *et al.*, 2005 a, b). After menopause, the vaginal microflora reverts to the pre-menarchal state (Brabin *et al.*, 2005 a, b); however, if post-menopausal women receive oestrogen replacement therapy the vaginal pH decreases and *Lactobacillus* spp. concentrations increase (Raz and Stamm, 1993). Previous studies monitoring IVF patients have identified a link between endogenous steroid hormones and presence of lactobacilli. Jakobsson and Forsum (2008) reported that women commencing IVF treatment who had vaginal microflora dominated by the lactobacilli, *L. crispatus*, *L. gasseri* or *L. jensenii* (species found in fertile women), maintained this flora for the duration of the cycle. In other women *L. delbruickii*, *L. rhamnosus, L. vaginalis, L. reuteri* or *L. iners* dominated the LGT at the commencement of the cycle, but as oestrogen levels rose, the vaginal flora altered, with the three major vaginal lactobacilli (*L. crispatus*, *L. gasseri* or *L. jensenii*) becoming predominant.

In a studies by Egbase *et al*. (1996, 1999), bacteria other than lactobacillus (including *Escherichia coli*, *Streptococcus* spp., other *Enterobacteriaceae*, *Staphylococcus* spp., *Haemophilus* spp., or mixtures of microorganisms − all of which can be part of the normal LGT regional flora) were recovered from culture of the catheter tips that transferred embryos to the uterus. Lower IVF pregnancy rates per embryo transfer were reported for patients with culture positive catheter tips (24.1%) compared to those for patients with culture negative catheter tips (33.3%) (Fanchin *et al.*, 1998). These findings also confirmed that mechanical transfer of normal regional flora of the female LGT to the female UGT occurred at the time of embryo transfer.

2.3.2 Vaginal/cervical bacteria and bacterial vaginosis

Changes that occur in the microbial LGT flora balance can result in bacterial vaginosis. An increase in LGT pH because of depletion of *Lactobacillus* spp. enables the overgrowth of other microorganisms. Eggert-Kruse *et al.* (2000) examined the antimicrobial properties of the cervical mucosa and found that lysozyme and immunoglobulin A played a major role in antimicrobial activity. Despite this relatively hostile environment for bacteria, bacterial vaginosis is a frequently encountered condition among women.

Vaginal smears are routinely Gram-stained to identify *Lactobacillus* spp. morphotypes and to determine the presence or absence of bacterial vaginosis in women. For women with bacterial vaginosis, the Gram stain shows a decrease or absence of lactobacilli. Women with bacterial vaginosis also have a thin homogenous, white vaginal discharge; elevated vaginal fluid pH > 4.5; a fishy odour is detected upon the addition of an amine to the vaginal secretions; and ‘clue cells’

are detected in a 'wet mount' microscopic examination of vaginal discharge (Hillier *et al*., 2008). Even though the Nugent score is considered the 'gold standard' for grading Gram-stained vaginal smears for the presence of *Lactobacillus* spp., *G. vaginalis*, Bacteroides and Mobiluncus morphotypes, this method fails to differentiate the various *Lactobacillus* spp. and other opportunistic pathogens present in low numbers within the vagina (Nugent *et al.*, 1991). Verhelst *et al*. (2005) proposed a modified criterion for grading Gram-stained vaginal smears to better quantify the different *Lactobacillus* spp. within the female LGT, as well as to identify other species, including *Bifidobacterium* spp., which also contribute to the LGT normal regional flora. Reports that are more recent suggest that a normal vaginal microenvironment in healthy women of reproductive age may result from colonisation by *Atopobium, Megasphaera* or *Leptotrichia* species flora, which are also lactic acid producing species. However, these reports did not investigate reproductive outcomes in these women (Verhelst *et al.*, 2005, Witkin *et al*., 2007).

In studies of over 2600 infertile women – both before and during ART treatment – the prevalence of bacterial vaginosis has been reported in the range of 4.2 – 40%, and is thought to be population dependent (Gaudoin *et al.*, 1999, Liversedge *et al.*, 1999, Moore *et al.*, 2000, Ralph *et al.*, 1999, Spandorfer *et al.*, 2001, Wilson *et al.*, 2002). In fertile women, bacterial vaginosis rates of 10-20% have been reported (Holmes *et al*., 2008). The following factors affect *Lactobacillus* spp. levels: antimicrobial treatment, hormonal imbalance, douching, use of non-barrier contraception, demographic factors (age and socioeconomic status), and the sexual history of the female including the age of commencement of sexual intercourse and the number of previous sexual partners (Witkin *et al*., 2007). Microorganisms that frequently

replace the normal lactobacillus dominant LGT flora include *Gardnerella vaginalis, Ureaplasma* spp., *Mycoplasma hominis, Streptococcus viridans* and anaerobic Gram-negative bacilli from the genera Prevotella, Porphyromonas, Bacteroides, Fusobacterium and Peptostreptococcus (Hillier *et al.*, 1993). Significantly, bacterial vaginosis has been associated with an increased risk of second trimester miscarriage (Oakeshott *et al*., 2002) and genital and obstetric infections including pelvic inflammatory disease (Catlin, 1992, Hay *et al.*, 1992), particularly in the presence of other sexually transmitted infections (Hillier *et al.*, 1996, Wiesenfeld *et al.*, 2002). However, the aetiology of bacterial vaginosis has not been fully investigated. Alterations of normal regional flora of the female LGT in women undergoing stimulated treatment cycles may be a cause of increased rates of bacterial vaginosis compared to rates reported for fertile women. Screening women undergoing ART for bacterial vaginosis prior to entry into ART cycles and at the time of oocyte retrieval would provide a more accurate record of the incidence of bacterial vaginosis and asymptomatic infections in this population (Wilson *et al.*, 2002). However, as systematic screening of the fertile population is not performed, a large population that may be used as a control group in the above study would be difficult to identify. Furthermore, the contraceptive pill reportedly provides protection against bacterial vaginosis, making the introduction of standardised screening unlikely (Riggs *et al.*, 2007).

2.3.3 Upper genital tract (UGT) infections

Ascending UGT infections occur when opportunistic pathogens present in the normal female LGT flora cause bacterial vaginosis and/or candidiasis and subsequently ascending invasive infections (Meirik, 2007, Holmes *et al*., 2008). Alternatively,

iatrogenic infections result from the transfer of microorganisms to the UGT by an invasive medical procedure or as a result of instrumental childbirth. During an ART treatment cycle there is also the risk of introducing microorganisms into the UGT at the time of oocyte retrieval (by trans-vaginal oocyte retrieval) or at embryo transfer (El-Toukhy and Hanna, 2006). However, these infections are rare, occurring in only 0.3% to 1.5% of women in studies of 17125 trans-vaginal oocyte retrievals (Bennett *et al*., 1993, Bergh *et al*., 1992, Dicker *et al*., 1993, Tureck *et al*., 1993). A previous history of pelvic inflammatory disease, tubal hydrosalpinges or drainage of ovarian endometriomas at trans-vaginal oocyte retrieval also enhances the risk of infection (Saltes *et al*., 1995, Tureck *et al*., 1993). Infection after trans-vaginal oocyte retrieval (requiring hospitalisation) has been documented for only 9/674 patients. Three of the nine women (all had no risk factors) received prophylactic antimicrobial treatment at the time of retrieval (Tureck *et al*., 1993). This suggests that prophylaxis does not significantly reduce the risk of infection. Even though the reported rate of infection following trans-vaginal oocyte retrieval without antimicrobial prophylaxis is very low at 0.6% (Bennett *et al*., 1993), complications following trans-vaginal oocyte retrieval are frequently associated with the antenatal period and late onset ovarian abscesses (Sharpe *et al*., 2006).

Infection of the female UGT can also potentially occur if microorganisms attach to the surface of motile spermatozoa, or if there are obligate intracellular parasites within the spermatozoa transported to the female UGT. *Ureaplasma* spp., *Mycoplasma spp, C. trachomatis, N. gonorrhoeae* and *E. coli* have all been shown to adhere to the surface of spermatozoa or form intracellular inclusions within the spermatozoa (Friberg *et al.*, 1987, James-Holmquest *et al.*, 1974, Keith *et al.*, 1984,

Law *et al*., 1988, Sanchez *et al.*, 1989, Svenstrup *et al.*, 2003, Wolner-Hanssen and Mardh, 1984) and may thus be transported to the female UGT where they may cause asymptomatic colonisation/infection or an inflammatory response.

2.3.4 The microflora of the endometrium, uterine tube and ovary

Accumulating evidence suggests that the female UGT may be asymptomatically colonised/infected (Holmes *et al*., 2008). Up to 19% of women reportedly have endometritis, a persistent inflammation of the endometrial lining (Farooki, 1967). Endometritis is often asymptomatic, but has been shown to reduce spontaneous and ART fertilisation and conception rates (Feghali *et al.*, 2003, Taylor and Frydman, 1996), potentially causing infertility. Furthermore, infectious inflammation of the endometrium has also been associated with preterm labour (Romero and Mazor, 1988) and preterm birth (Gravett *et al.*, 2000).

Endometritis is frequently a polymicrobial infection caused by ascending invasive infections by endogenous microorganisms or STIs. Endometritis has been reported in association with a diagnosis of pelvic inflammatory disease (Paavonen *et al*., 2008) but also in the absence of this infection (Lucisano *et al.*, 1992). Jacobsson *et al*. (2002) studied a Swedish cohort of 924 women and found that the risk for post partum endometritis tripled for women with bacterial vaginosis in early pregnancy compared to patients with normal vaginal flora. There is also a risk of introducing microorganisms into the endometrium during gynaecological procedures as well as procedures for infertility investigations and treatment, potentially leading to endometritis (Kiviat *et al.*, 1990 a, b).

The uterine tubes play an integral role in reproduction and undergo cyclical changes in morphology and ciliary activity that are dependent on ovarian hormones (Lyons *et al*., 2006). Recent reviews have reported that infection reduces ciliary motion and even destroys cilia within the uterine tubes (Lyons *et al*., 2006). Reduced ciliary function can be a cause of infertility and can result in ectopic pregnancy since the embryo relies on cilia to facilitate its propulsion through the uterine tubes into the uterus. In addition, inflammation of the lumen of the uterine tubes results in tubal occlusion and tubal factor infertility (Lyons *et al*., 2006). A single chlamydial infection does not result in tubal scarring (Patten, 1987); however, prolonged exposure to *C. trachomatis* due to a chronic persistent infection or frequent re-infection has been associated with an autoimmune response to chlamydial heat shock protein (which shares homology with human heat shock protein) and the chronic inflammation associated with tubal factor infertility (Brunham and Peeling, 1994, Mardh, 2004, Ness *et al.*, 2008). The severity of the inflammatory response to chlamydial infection is enhanced during re-infection, causing inflammation, tissue damage and scarring (Stephenson *et al.*, 2003). Recent studies by Hvid *et al*. (2007) have concluded that damage to the uterine tubes is disproportional to the number of *C. trachomatis* infected cells, suggesting that uterine tube endothelial cell lysis did not occur as a direct result of infection. In their study, they instead demonstrated a toxic effect by IL-1 on ciliated uterine tube cells.

There have been very few reports of ovarian infections in the literature: however, tubo-ovarian abscesses have been associated with pelvic inflammatory disease in up to 30% of women hospitalised for this infection (Landers and Sweet, 1983); but ovarian infections have rarely been reported following inflammatory bowel disease

(Hartmann *et al.*, 2009). Infection of the ovary can also be a complication of obesity, urinary tract infection, constipation and poor hygiene (Hartmann *et al.*, 2009). Tubo-ovarian abscess formation after haematogenous spread of *M. pneumoniae* from a primary respiratory tract infection has also been reported (Goulet *et al.*, 1995 a). Similarly, fungal infections have caused tubo-ovarian abscesses after a primary respiratory infection and then subsequent haematogenous spread and infection of the female UGT (Kepkep *et al.*, 2006) (See section 2.5.2).

At the time of ART oocyte retrieval, the clinician aspirates follicular fluid from ovarian follicles. The follicular fluid can subsequently be tested for antimicrobial activity by agar diffusion and broth dilution antimicrobial sensitivity assays (Stepanovic *et al*., 2003). Alternatively, filtered follicular fluid can be inoculated with several species of microorganisms and then monitored for bacterial growth (Gurgan *et al.*, 1993). These experiments revealed that follicular fluid has antimicrobial activity against some Gram-positive bacteria, most likely due to the presence of lysozyme (Stepanovic *et al*., 2003). Conversely, follicular fluid has also been shown to support the growth of some Gram-negative pathogens and *Candida* spp. (Gurgan *et al.*, 1993). Based on these findings, it can be concluded that follicular fluid may support the growth of microorganisms *in vivo*.

Regardless of how microorganisms gain access to the UGT, infection can result in pelvic inflammatory disease an infectious, inflammatory disorder of the female UGT (Holmes *et al*., 2008). Long-term sequelae of pelvic inflammatory disease include tubal infertility, ectopic pregnancy, pelvic pain and tissue destruction within the UGT (Cherpes *et al.*, 2006).

2.4 Male reproductive tract microbiology

2.4.1 Male urogenital tract normal regional flora

The distal urethra of the male LGT is frequently colonised by anaerobic bacteria, including *Bacteroides* spp. and Gram-positive facultative bacteria such as *Staphylococcus* spp. (Spaine *et al.*, 2000, Woolley, 2000). Similar to follicular fluid and cervical mucus, human semen demonstrates antimicrobial activity aided by the seminal plasma components of spermatozoaine, lysozyme, lactoferrin and protease inhibitors: including secretory leukocyte protease inhibitor and cystatin (Bourgeon *et al.*, 2004) as well as mucins and defensins (Halling *et al.*, 2008). These agents exhibit broad antimicrobial activity against both Gram-positive and Gram-negative bacteria, viruses and some fungi (Bourgeon *et al.*, 2004) (See Appendix 1. Host defence proteins expressed in the male reproductive tract).

Semen can become colonised/infected at the time of ejaculation with the normal regional flora from the urethra and the perineum and/or the gastrointestinal tract. These microorganisms are often opportunistic pathogens (Landgren *et al.*, 1984, Toth and Lesser, 1981) capable of causing either symptomatic or asymptomatic genital tract infections (Stone, 1980). Liversedge *et al.* (1996) and Bussen *et al.* (1997) found no correlation between the presence of bacteria in semen and fertilisation rates, cleavage rates of the embryo, or pregnancy rates after IVF insemination. Conversely, decreased fertilisation rates and embryonic development rates were reported after IVF insemination if bacteria were detected in semen that had undergone an IVF washing procedure when compared to the original untreated semen sample. If bacteria remained adherent to washed spermatozoa, then decreased fertilisation rates were also observed when compared to spermatozoa with no evidence of bacterial

attachment (Guillet-Rosso *et al.*, 1987). The ability of some species to attach to and invade spermatozoa (Friberg *et al.*, 1987, James-Holmquest *et al.*, 1974, Keith *et al.*, 1984, Law *et al.*, 1988, Sanchez *et al.*, 1989, Svenstrup *et al.*, 2003, Wolner-Hanssen and Mardh, 1984), and to remain adherent following standard semen washing procedures (Knox *et al.*, 2003), makes it difficult to separate bacteria from semen and seminal fluid prior to ART insemination procedures.

During pregnancy, the cervix and its mucosal plug act as physical barriers between the vaginal flora and the uterus. However, during uterine contractions, rapid transport of LGT secretions (including microorganisms from the vagina) to the UGT can occur (Barnhart *et al.*, 2001). In animal model studies, immotile spermatozoa were artificially inseminated to determine if these spermatozoa were transported into the UGT. Immotile spermatozoa were detected in the uterus shortly after insemination (Drobnis and Overstreet, 1992). Rapid transport of the spermatozoa to the UGT occurred during uterine contractions, demonstrating that spermatozoa can be immotile due to bacterial infection and can still gain access to the uterine cavity. This also implies that spermatozoa with adherent or intracellular bacteria or viruses (irrespective of their motility status) can be transported into the female UGT by uterine contractions. These spermatozoa may also potentially infect the oocyte at fertilisation. Alternatively, microorganisms adherent to spermatozoa may gain access to the female UGT and then colonise/infect the endometrium. Furthermore, Toth *et al.* (1982) demonstrated that some microorganisms (*Staphylococcus* spp., *Streptococcus* spp., *E. coli, Neisseria* spp., *Klebsiella* spp., *Peptostreptococcus* spp., *Propionibacterium* spp., *Fusobacterium* spp., *Proteus* spp., *Pseudomonas aeruginosa*, *Enterobacter aerogenes* and *Salmonella enteritidis*) can migrate through

the cervical mucus attached to motile spermatozoa. These studies confirm that seminal fluid containing either live or dead spermatozoa can be a source of female UGT colonisation/infection.

2.5 Upper genital tract infections

2.5.1 Bacterial infections of the female and male UGT

2.5.1a Genital mycoplasmas

Many bacterial UGT pathogens are normal regional flora of the LGT that have ascended and caused UGT infection. The human *Ureaplasma* spp., *U. urealyticum* and *U. parvum*, are part of the normal LGT regional flora in 40–80% of women, but LGT colonisation with *Ureaplasma* spp. is not associated with adverse pregnancy outcomes (Cassell *et al.*, 1993). Ureaplasmas are also present in the normal regional flora in up to 50% of males (Shepard, 1974). *Ureaplasma* spp. have been implicated in bacterial vaginosis, pelvic inflammatory disease, endometritis (Kanakas *et al.*, 1999) and spontaneous abortion (Naessens *et al.*, 1986, Quinn *et al.*, 1983). Witkin *et al.* (1999) used PCR assays to detect ureaplasmas in the uterine cervix (LGT) and found that 17.2% of clinical specimens were ureaplasma-positive; however, ureaplasmas were not detected in the UGT specimens collected from these same women. In contrast, tests on pregnant women found ureaplasmas in the LGT of 57% of participants and in 19% of placentas (Knox and Timms, 1998). For some of the tested women, different ureaplasma serovars were isolated from the LGT and the infected placenta, which indicates that the LGT and UGT can be separately colonised and suggests that the source of UGT microorganisms is not necessarily the female LGT (Knox and Timms, 1998). Furthermore, in a study conducted in association with the Wesley IVF Service (Brisbane, Australia), the presence of ureaplasmas in

washed semen was associated with reduced viable pregnancy rates for couples undergoing ICSI treatment, as well as increased miscarriage rates after insemination by either IVF or ICSI, compared to couples with ureaplasma negative washed semen (Knox, unpublished data). The ureaplasmas are opportunistic pathogens (Taylor-Robinson, 1996) that cause various infections in the urogenital tracts of males and females.

Ureaplasma spp. have also been isolated in the endometrium of non-pregnant women (Kanakas *et al.*, 1999). Ureaplasmas colonising the endometrium, either by ascending to the female UGT (from the female LGT) or by adherence to spermatozoa or within semen, can colonise/infect the chorioamnion and amniotic fluid of pregnant women (Cassell *et al.*, 1993). Altered host immune responses can occur as a result of bacterial vaginosis or STIs, potentially further facilitating the ascension of opportunistic LGT microorganisms to the UGT (Holst *et al.*, 1994, McGregor *et al.*, 1990). In a study of 83 women, Martínez *et al*. (2001) found that 28% of patients with positive amniotic fluid ureaplasma cultures also had bacterial vaginosis.

In women with endometritis, the presence of *Ureaplasma* spp. has been associated with infertility (Kanakas *et al.*, 1999). There are also rare reports of tubal infertility associated with ureaplasma pelvic inflammatory disease (Henry-Suchet *et al.*, 1980). ART-specific studies are not numerous; furthermore, their findings are inconsistent. Shalika *et al*. (1996) reported decreased fertilisation rates when ureaplasmas were present in semen at the time of IVF, whilst others have shown that ureaplasmas do not affect fertilisation rates (Hill *et al.*, 1987). Montagut *et al*. (1991) suggested that

embryo implantation may be impaired if embryos were transferred to a woman with asymptomatic ureaplasma endometritis.

The presence of *M. hominis* in the reproductive tract is estimated to occur in 4 – 17% of sexually active adults (Knox *et al.*, 2003, Spicer, 2000), and can cause ciliostasis (no movement) and swelling of uterine tube cilia (Mardh and Westrom, 1970). Similarly, *M. genitalium* infections have been reported in 4.5% of females and 9.6% of males (Jensen *et al.*, 2003). Whilst Debattista *et al.* (2004) found no evidence of *M. hominis* in uterine tubes in their Australian study population; both *M. hominis* and *M. genitalium* have been detected in the female UGT and uterine tubes in studies by other authors (Cohen *et al.*, 2005, Heinonen and Miettinen, 1994, Stagey *et al.*, 1992). *M. genitalium* was isolated from 21.1% of men with non-gonococcal urethritis (Jensen *et al.*, 2003); and both *M. genitalium* and *M. pneumoniae* have been detected in the LGT of sexually active women (Palmer *et al.*, 1991, Taylor-Robinson *et al.*, 1993 b). Serological testing of serum has confirmed an association between mycoplasmas and cases of pelvic inflammatory disease (Moller *et al.*, 1985). As only *M. hominis* can be cultured easily, *M. genitalium* and *M. pneumoniae* can best be identified by PCR detection of these microorganisms in UGT tissues. However, *M. genitalium* appears to be associated with a low risk of adverse pregnancy outcome (Labbe *et al.*, 2002, Oakeshott *et al.*, 2004). Further research to investigate the prevalence of *Mycoplasma* spp. and associations with adverse pregnancy outcome are now possible using molecular techniques.

2.5.1b Chlamydia trachomatis

C. trachomatis, an obligate intracellular parasite, infects the epithelial cells lining the genital tracts of the human host (Barron *et al.*, 1984). *C. trachomatis* can cause ascending infection resulting in tubal infertility (Swenson *et al.*, 1983, Swenson and Schachter, 1984), ectopic pregnancy (Wolner-Hanssen, 1990) and is associated with an increased risk of developing invasive squamous cell carcinoma of the uterine cervix (Koskela *et al.*, 2000). *C. trachomatis* infections in women are often asymptomatic. In contrast, in men, chlamydia may cause dysuria and urethral discharge (Ingalls *et al.*, 1995). The reported effects of chlamydial infection on human spermatozoa are inconsistent. Vigil *et al*. (2002 b) concluded that spermatozoa function does not appear to be affected by existing or previous chlamydial infection; however, female tubal infertility is common in partnerships where chlamydial antibodies are found in either partner (Eggert-Kruse *et al.*, 1990). In contrast, other studies have reported a decrease in the percentage of motile spermatozoa, and an increase in the proportion of dead spermatozoa following co-incubation with either *C. trachomatis* or chlamydial lipopolysaccharide (Hosseinzadeh *et al.*, 2001). DNA fragmentation, a marker of apoptosis (programmed cell death), has also been assessed following natural genital tract infection by *C. trachomatis,* and the authors concluded that infection by *C. trachomatis* may induce DNA damage (Satta *et al.*, 2006). It has been proposed that spermatozoa with damaged DNA may be selected for IVF because DNA damage does not result in immediate cell demise, and successfully fertilise an oocyte; however, depending on the extent of DNA damage, these embryos may fail to develop and result in a successful pregnancy (Aitken, 1999). Therefore, it was postulated that *C. trachomatis* infection of spermatozoa acts at post-fertilisation

stages, since it does not inhibit spermatozoa-zona pellucida binding, follicular fluid induced acrosomal binding, or oocyte penetration assays (Wolff *et al.*, 1991). *C. trachomatis* infection has a direct cytotoxic affect on the uterine tube mucosa, causing loss of microvilli and disruption of cell junctions due to rupture of epithelial cells (Cooper *et al.*, 1990), often resulting in scarring, tubal occlusion and infertility. As previously discussed, chlamydial disease and damage to the female UGT may also result from delayed hypersensitivity reactions to human heat shock proteins (Morrison *et al.*, 1989). There is a correlation between the level of the host immune response to chlamydial heat shock protein and the extent of tubal damage (Eckert *et al.*, 1997).

Vigil *et al.* (2002 b) performed *in vitro* studies to assess the possible relationship between chlamydia-infected oocytes and early pregnancy loss and found that *C. trachomatis* was capable of penetrating the cytoplasm of the ovum. After incubating *C. trachomatis* infected human semen (from infertile men) with zona-free hamster oocytes and fertilisation occurred, C. *trachomatis* was observed within the cytoplasm of the hamster oocytes by scanning electron microscopy and transmission electron microscopy (Vigil *et al.*, 2002 a). The presence of *C. trachomatis* was also confirmed within hamster oocytes using monoclonal antibodies to the chlamydial major outer membrane protein, which is expressed in the 15 known *C. trachomatis* serotypes in both elementary bodies and reticulate bodies (Vigil *et al.*, 2002 a). This animal model finding is unique, since no other bacterial species have been reported within oocytes.

Vigil *et al*. (2002 b) proposed two mechanisms by which *C. trachomatis* infection may induce first trimester spontaneous abortion: firstly, the embryo may abort because of direct infection of the zygote as a result of fertilisation by a *C. trachomatis* infected spermatozoon resulting in zygote lysis or chronic blastomere infection; and secondly, the embryo may be targeted by a maternal immune response to human heat shock protein. This may occur if the mother had previously been exposed to chlamydial heat shock protein (Witkin *et al*., 1999), again resulting in cross reactivity with conserved human heat shock protein 60 epitopes produced by the embryo five days post-ovulation. Since the work of Vigil *et al*. (2002 b) demonstrated reticulate bodies of *C. trachomatis* inside hamster oocytes, which were incubated with infected human spermatozoa, this supports the hypothesis that spermatozoa may infect the zygote during fertilisation or act as facilitators in the transport of these bacteria to the female UGT, potentially resulting in spontaneous abortion or embryo resorption (Witkin *et al*., 1999). In natural pregnancies, once *C. trachomatis* infects the female UGT, there is also the potential for the embryo to become infected/colonised after ovulation and whilst travelling through the uterine tubes to the uterus (Vigil *et al*., 2002 b). In conclusion, outcomes of both assisted and natural pregnancies are potentially affected by *C. trachomatis*, regardless of its origin and mode of introduction to the female UGT. These findings suggest the need for further study of this species in relation to fertility.

2.5.1c Neisseria gonorrhoeae

Neisseria gonorrhoeae is an STI associated with long-term sequelae. Males experience symptoms of painful urination and purulent urethral discharge within days of exposure to *N. gonorrhoeae*, which can lead ultimately to epididymitis

(Handsfield, 1983). In contrast, early gonococcal disease is often asymptomatic in females, but may include painful urination, purulent urethral or vaginal discharge or acute abdominal pain due to pelvic inflammatory disease. As the infection ascends into the UGT it may cause endometritis, pelvic peritonitis, salpingitis and tubo-ovarian abscess, if not treated early during the infectious period (Hook and Handsfield, 2008). Infection in the uterine tubes with *N. gonorrhoeae* or exposure to lipopolysaccharide can result in cessation of ciliary activity but not necessarily ultrastructural damage (Mardh *et al*., 1979). However, gonococci can also invade the non-ciliated cells of the uterine tube mucosa, destroying the ciliated cells by causing sloughing (McGee *et al.*, 1983). As with the chlamydiae, *Neisseria* spp. have been found to inhibit apoptosis in cells, and promote bacterial survival (Dean and Powers, 2001).

Bessen and Gotschlich (1986) proposed that the pathogenicity of *N. gonorrhoeae* is dependent on both the presence of pili and the opacity-associated protein II. Clinical isolates of *N. gonorrhoeae* have been shown to selectively infect different anatomical sites. Following attachment, the gonococci are able to penetrate the mucus-secreting columnar epithelium of the urethra, cervix and uterine tubes (McGee *et al.*, 1983), resulting in activation of the host immune response (Meyer, 1999).

Invasive infections ascending from the LGT account for most cases of serious morbidity associated with *N. gonorrhoeae*, including salpingitis, epididymitis and bacteraemia (Handsfield, 1983). In untreated gonorrhoea, 10-17% of women will acquire an UGT infection or pelvic inflammatory disease. The antimicrobial properties of the cervical mucus sometimes prevent ascending infection; however,

during menstruation, the cervical plug disintegrates and gonococci are able to travel to the UGT and can cause infection and long term sequelae, including infertility (Holmes *et al*., 2008).

In pregnant women, untreated gonorrhoea is associated with preterm delivery, preterm labour or rupture of membranes and septic abortion (Amstey and Steadman, 1976, Sarrell and Pruett, 1968). After the 12th week of gestation, the chorion attaches to the endometrial decidua, blocking the ascending spread of gonococci, whereby the chorioamnion becomes the potential site of infection (Quinn *et al.*, 1987).

2.5.1d Gram-positive cocci

Streptococci, staphylocci and enterococci colonise both the female LGT and the male urethra, and in males can cause prostatitis and epididymitis (Weidner *et al.*, 1999). A differentiating feature between the three cocci is that whilst infection of the male genitourinary tract with enterococci results in abnormal semen andrology parameters (Mehta *et al.*, 2002), the presence of streptococci and staphylococci within the urethra of infertile men has no effect on semen characteristics (Rodin *et al.*, 2003, Virecoulon *et al.*, 2005). *Enterococcus* spp., *Staphylococcus* spp., and *Streptococcus* spp. can also be isolated from female UGT infections, including pelvic inflammatory disease (Saini *et al.*, 2003) and chronic endometritis (Cicinelli *et al.*, 2009). Enterococci and streptococci have also been isolated from internal organs of stillborn infants (Tolockiene *et al.*, 2001). It has been suggested that these species are so pathogenic that after infection of the foetal tissues, the foetus has insufficient time to mount an immune response, resulting in its demise (Christensen *et al.*, 1982, McClure and Goldenberg, 2009). Studies have also shown that a polymorphism in

the IL-1 receptor antagonist (ra) gene can inhibit the foetal immune response (Gerber *et al.*, 2005). Evidence of an immune response to infection is measured by inflammation in the foetal tissues the cord and the chorioamnion (Pacora *et al.*, 2002). It has, therefore, been proposed that foetuses responding to infection produce a pro-inflammatory cascade initiating pre-term labour and the foetus is aborted from the infected intrauterine environment (Romero *et al.*, 2007).

2.5.1e Gram-negative bacilli

E. coli is the bacterium isolated most frequently from male urogenital infections of the prostate (Weidner *et al.*, 1999) and epididymis (Weidner *et al.*, 1987). In addition, *E. coli* is also isolated from ejaculates of asymptomatic, infertile men (Eggert-Kruse *et al.*, 1995). *E. coli* is primarily a commensal of the gastrointestinal tract and is found in the anterior urethra of males as well as the lower urethra, the perineum and the LGT of females. *E. coli* is one of the microorganisms frequently isolated from infected placental tissue (Wilson, 2005) and from within the chorioamnion of placentas of women who deliver preterm (Hillier *et al.*, 1988, Splichalova *et al.*, 2005). It is also one of the most prevalent aetiological agents isolated after stillbirths (Tafari *et al.*, 1976), whereby culture of foetal tissues from stillborn infants identified both *E. coli* and *Klebsiella* spp. (Axemo *et al.*, 1993). *E. coli* forms part of the normal regional flora of the female gastrointestinal tract and LGT, and it is also often isolated from tissue samples obtained from women with post partum endometritis (Watts *et al.*, 1990). Interestingly, endometritis occurs more often after Caesarean delivery than after normal vaginal delivery.

Klebsiella spp. are frequently isolated from women with pelvic inflammatory disease (Saini *et al.*, 2003). *Pseudomonas aeruginosa* is also a causal agent of pelvic inflammatory disease in females and epididymitis and prostatitis in males (King *et al.*, 2002). Studies of infertile couples have also revealed that the presence of the Enterobacteriaceae, *E. coli, Klebsiella* spp. or *Proteus* spp., on embryo-transfer catheter tips was correlated with the significantly lower pregnancy rates for these couples when compared to couples with an absence of these microorganisms (Egbase *et al.*, 1996).

2.5.1f Other enteric bacteria

Helicobacter pylori, another gastrointestinal tract pathogen, has also been under investigation as a potential cause of infertility. Recent studies have shown that this bacterium and human spermatozoa share similar antigenic epitopes (Kurotsuchi *et al.*, 2008). Experiments investigating the implications of these similarities found that *H. pylori* co-incubated with spermatozoa had no effect on spermatozoon motility or sperm counts. However, further investigations are examining the possibility that anti-*H. pylori* antibodies in vaginal secretions and follicular fluid may react with spermatozoa (Figura *et al.*, 2002).

2.5.1g Anaerobic species

Anaerobic microorganisms: *Bacteroides* spp., *Peptostreptococcus* spp., *Fusobacterium* spp., *Prevotella* spp., *Propionibacterium* spp., and *Bifidobacterium* spp. are frequently implicated in genital tract infections and they may be asymptomatic colonisers of endometrial tissues (Onderdonk *et al.*, 2008 a, b, Tolockiene *et al.*, 2001, Viniker, 1999). It has been reported that within the genital

tract anaerobes outnumber aerobes in a 10:1 ratio. Anaerobic species are frequently isolated in cases of acute pelvic inflammatory disease (Saini *et al.*, 2003); and these infections are commonly polymicrobial, which may enhance pathogenicity (Eschenbach *et al.*, 1975). The reactivation of pelvic inflammatory disease has been observed in women with prior UGT damage, and even more frequently in women with a history of UGT infection, pelvic adhesions, endometriosis or persistent bleeding, or haematomas that provide a cultural niche for bacteria (El-Shawarby *et al.*, 2004). It has also been reported that if anaerobic bacterial vaginosis-associated microorganisms are cultured from the female LGT, then these women have a greater propensity for developing pelvic inflammatory disease (Ness *et al.*, 2005).

Mobiluncus curtisii and *Mobiluncus mulieris*, microorganisms frequently isolated from women with bacterial vaginosis have been shown to produce cytotoxins, which cause loss of cilia, and bloating and detachment of ciliated cells from within the uterine tubes (Taylor-Robinson *et al.*, 1993 a). Similarly, *Bacteroides ureolyticus*, also associated with bacterial vaginosis, releases lipopolysaccharide causing sloughing of cells and loss of ciliary activity in the uterine tube mucosa (Fontaine *et al.*, 1986).

Actinomyces spp. are normal inhabitants of the gastrointestinal tract and they are thought to gain access to the female UGT via endogenous or iatrogenic routes: ascending infection; intrauterine device usage (up to 11.6% of users); post-surgery/appendectomy; or following bowel perforation (Evans, 1993). These bacteria do not normally cross the mucosal barrier, except due to injury − such as in iatrogenic infections, or when they are found in the presence of other

microorganisms. *Actinomyces* spp. are often found alongside fusiform, spindle-shaped bacilli (*Fusobacterium* spp.), Gram-negative bacilli and *Streptococcus* spp. (Fiorino, 1996). *Actinomyces* spp. has been detected in the UGT of both men and women and can cause pelvic inflammatory disease, prostatitis and pelvic and abdominal abscesses, with infection occurring more often in males than in females (Urbina *et al*., 2006).

The spirochetes, *Treponema pallidum*, *Borrelia burgdhoferi* and *Leptospira interrogans*, are also able to migrate through the UGT and cross the placental barrier, resulting in stillbirth (McClure and Goldenberg, 2009) or post partum infantile death (Schlesinger *et al*., 1985). Antimicrobial therapy does not ensure an improved pregnancy outcome, often failing to prevent neural and cardiac tissue infection and subsequent damage within the neonate (Walsh *et al*., 2006).

2.5.2 Fungal UGT infections

Fungal infections in the UGT are rare; they are more commonly detected in other tissues, the respiratory tract being the most predominant site for primary fungal infections. Fungal peritonitis has been reported as part of a polymicrobial infection of aerobes and anaerobes, cultured from aspirated ascites (Mikamo *et al.*, 2003).

Cryptococcus spp. has been isolated from a compact inflammatory mass within a uterine tube, with evidence of granulomatous inflammation involving the luminal surface and muscle wall (Plaut, 1950). This microorganism has also been isolated from faeces, urine and the surface of the vulva in healthy individuals, and therefore it can gain access to the UGT from these endogenous sites (Plaut, 1950).

In 1970, Craig *et al*. described a case of sexually transmitted blastomycosis. The male partner was diagnosed with disseminated infection by *Blastomyces dermatitidis*, involving the epididymis, prostate and testes (Craig *et al.*, 1970). Soon after diagnosis, his female partner presented with endometrial, uterine tube and perineal blastinomycosis infection. This was the first reported case of sexually transmitted blastomycosis infection. However, there had been earlier reports of infection of the genitourinary system by this organism (Busey, 1964), after inhalation of fungal spores followed by haematogenous dissemination, or alternatively reactivation of a latent infection.

Coccidioides immitis is also a causative agent of pelvic inflammatory disease, which appears to occur after primary respiratory infection (Saw *et al.*, 1975). The peritoneal cavities of over 50 women were examined by laparotomy and multiple miliary coccidioidomas, tubo-ovarian abscesses, adhesions and tissue necrosis of the ovaries and uterine tubes were observed. Such extensive inflammation was associated with failure to conceive, miscarriage and even infant death due to systemic coccidioidal infection at birth. In most extreme cases it even necessitated total hysterectomy following diagnosis (Saw *et al.*, 1975, Smale and Waechter, 1970).

Aspergillosis infection is the third most common opportunistic fungal pathogen in immunocompromised patients or those with a malignancy (Powell *et al.*, 1998). *Aspergillus* spp. has been identified as the causative organism of a tubo-ovarian abscess in a renal transplant patient (Kim *et al.*, 2001) and of pelvic inflammatory disease in women with a history of intrauterine device usage (Kostelnik *et al.*, 1976). Presence of *Aspergillus* spp. in males has been detected in iatrogenic infection of the

prostate (Abbas *et al.*, 1995, Fisher *et al.*, 1998) and the testes (Singer *et al.*, 1998) and also in opportunistic infections of the scrotum (Powell *et al.*, 1998).

Candida spp. can also infect the UGT and have been implicated in infections in immunocompromised hosts, causing fungaemia, urinary tract infection, peritonitis and chorioamnionitis (Sobel, 1999). *C. glabrata* has been isolated from an invasive uterine mole (Mikamo *et al.*, 2003), and there have been reports of *C. albicans* isolated from neonates after stillbirth (McClure and Goldenberg, 2009). *Candida* spp. are opportunistic pathogens, gaining access by an endogenous route and causing ascending invasive infections. Studies have been performed to investigate the potential for an altered immune response to genital tract challenge by *Candida* spp. in women with recurrent vulvovaginal candidiasis. Fidel *et al*. (1997) concluded that women with recurrent candidiasis had decreased levels of IL-12 and IL-5 in cervicovaginal secretions that may lead to altered humoral and cell-mediated immune responses.

Nocardia spp. infection is very rare in the immunocompetent host (Dikensoy *et al.*, 2004) and primary infection is thought to occur in the respiratory tract with dissemination in the host as a result of immunosuppression or pregnancy (Kepkep *et al.*, 2006). In the case history reported by Kepkep *et al.* (2006), *Nocardia* spp. caused a tubo-ovarian abscess in a pregnant woman, which resulted in a foul smelling vaginal discharge, urinary tract infection, decreased amniotic fluid volume, retroplacental haematuria and finally miscarriage at 13 weeks gestation. Further investigations found that the patient had adhesions of the uterus to the rectosigmoid

and posterior wall of the pelvic peritoneum and to the appendix and also had bilateral pleural effusions as a result of infection.

2.5.3 Parasitic infections of the UGT

Parasitic infections of the UGT with organisms other than *Trichomonas vaginalis* are uncommon. *T. vaginalis* has been reported as a cause of asymptomatic genitourinary infections in men and painful acute endometritis and genitourinary infections in women (Cherpes *et al.*, 2006). Trichomonads have been isolated from the uterine tubes and peritoneal cavity in women with acute salpingitis, which can result in tubal occlusion and tubal infertility (Mardh and Westrom, 1970).

In rare cases, adult worms and ova of *Enterobius vermicularis* have been isolated in the female UGT and peritoneal cavity, usually in asymptomatic infections; although there have been six documented cases where *E. vermicularis* infection has caused a significant inflammatory response in the host (Saffos and Rhatigan, 1977). Diagnosis of infections in the peritoneal cavity of women suggests that these parasites can migrate through the LGT and UGT and into the uterine tubes and reach the peritoneal cavity (Symmers, 1950). A severe inflammatory response may result in tubal occlusion and tubal factor infertility.

Goverde *et al.* (1996) reported a case of *Mansonella perstans* microfilariae in the follicular fluid of a woman who also had *Schistosoma haematobium* ova isolated in her blood and faecal cultures. The patient also had a history of hepatitis A, *Plasmodium ovale* and *P. falciparum* infections. Investigations of a urinary tract infection and skin ulcers associated with schistosome and filarial infection were

initially negative. However, subsequent testing isolated live *M. perstans* from the follicular fluid and blood. Past chlamydial infection was considered the primary cause of tubal infertility in this patient. This study, however, did not offer any suggestions as to the role of *M. perstans* in infertility, as normal fertilisation and cleavage occurred in the ART treatment cycle of the woman who was infected with this parasite (Goverde *et al*., 1996). It may be that infection of the endometrial tissue prevented implantation by activation of the host immune response. At present, there is no effective treatment for *M. perstans*.

Over 200 cases of female genital tract infection by *Schistosoma* spp. have been reported in the literature (Gelfand, 1971). Schistosomiasis causes granulomatous inflammatory processes, resulting in peritubal adhesions, tubal occlusion and decreased fertility (Balasch *et al*., 1995). Parsons and Sommers (1978) proposed that *Schistosoma* spp. spread to the UGT via the ovarian veins, allowing the parasites to lodge in the uterine tubes and ovaries and deposit their ova. This is supported by the finding that in 62% of women with bladder infestation, ova were also detected in the uterine tubes (Bland and Gelfand, 1970).

Plasmodium spp. infection is often asymptomatic and the risk of infection is increased by a positive HIV status (Desai *et al.*, 2007). Persistent *Plasmodium* spp. infection of the placental membranes in pregnant women has resulted in miscarriage and stillbirth or long-term sequelae (if infants survived beyond the perinatal period), such as parasitaemia, low birth weight and intra-uterine growth retardation (van Geertruyden *et al*., 2004). Antiparasitic treatment has been shown to decrease adverse pregnancy outcomes (Villegas *et al.*, 2007).

These reports demonstrate the wide variety of aetiological agents that can be isolated from both male and female UGT, also proving that infections of the UGT are frequently polymicrobial in nature. Many of these case histories represent rare events, and some of these microorganisms are not globally endemic. Nevertheless, microbiologists should be alerted to the possible diverse infections within the genital tract. Their treatment and prevention is necessary in order to prevent potentially adverse effects of such pathogens on fertility, pregnancy and subsequent foetal and infantile outcomes.

2.6 Sexually transmitted infections (STIs)

Microorganisms causing STIs are not typically part of the normal regional flora, with the exception of *Ureaplasma* spp. and *Mycoplasma* spp., which are acquired through sexual activity and can colonise or cause genital tract infections. *C. trachomatis, N. gonorrhoeae, Treponema pallidum, T. vaginalis* and viruses including human immunodeficiency virus (HIV), hepatitis B and C viruses (HBV, HCV), human papilloma virus (HPV) and herpes simplex viruses (HSV) (Pertel and Spear 2008, Corey and Wald, 2008) can all cause STIs. STIs may be symptomatic, but as previously discussed, often elicit no symptoms, particularly early in infection, and thus usually remain untreated. Untreated STIs can cause ascending invasive infections, which can result in damage to the female or male UGT, an inflammatory immune response that causes further damage to the UGT, scar tissue formation or tubal occlusion, pelvic inflammatory disease in females and epididymitis in males (Berger, 1999, Westrom and Eschenbach, 1999). STIs of the genital tract may also be present as persistent or unresolved infections, which can be reactivated later; this can be true for chlamydial (Schachter, 1999) or herpes infections, HSV, Epstein-Barr

virus and cytomegalovirus infections (Drew and Bates, 1999, Pertel and Spear, 1999, Slobodand Sixby, 1999). Long-term sequelae of STIs may result in male and female infertility or adverse pregnancy outcomes. Vertical transmission and infection of the foetus *in utero* has also been reported (Lefevre *et al.*, 1988).

2.7 Bacteria that adhere to the surface of spermatozoa

Of the large number of microbial species documented as commensals, pathogens or STI agents within the male and female genital tracts, only five genera have been reported to adhere to the surface of spermatozoa: *Ureaplasma* spp., *Mycoplasma* spp., *E. coli*, *C. trachomatis*, and *N. gonorrhoeae.* Spermatozoa may therefore be the vector that transports these microorganisms to the female UGT. Significantly, each of these five bacteria has been associated with UGT infections in both pregnant and non-pregnant women, as well as compromised fertility and adverse pregnancy outcomes.

2.7.1 Genital Mycoplasmas

Several microscopic studies have demonstrated the presence of ureaplasmas on the surface of spermatozoa (Knox *et al.*, 2003, Reichart *et al.*, 2000, Talkington *et al.*, 1991). Lingwood *et al.* (1990 a) found that ureaplasmas bind to sulphogalactoglycerolipid *in vitro* and since sulphogalactoglycerolipid is also present on all male mammalian germ cell membranes, it has been proposed that sulphogalactoglycerolipid is the binding receptor for ureaplasmas adherent to spermatozoa (Lingwood *et al.*, 1990 a, b). *Ureaplasma* spp. can strongly adhere to the surface of spermatozoa and even remain adherent to spermatozoa following ART semen washing procedures prior to ART treatment (Knox *et al.*, 2003) it was

therefore proposed that these microorganisms may gain access to the female UGT by adherence to the surface of spermatozoa.

2.7.1a Ureaplasma spp

The role of ureaplasmas in infertility and adverse pregnancy outcomes is gaining importance as a research subject, as evidence emerges to suggest that ureaplasma adherence to spermatozoa can also enhance spermatozoa motility (Talkington *et al*., 1991, Reichart *et al*., 2000, Knox *et al*., 2003). It has been proposed that spermatozoon motility is enhanced by the release of ATP during the metabolic degradation of urea (accumulated in cell membranes) by ureaplasmas (Reichart *et al*., 2000). This may facilitate ureaplasma access to the female UGT, thereby enhancing the invasiveness of *Ureaplasma* spp. (Reichart *et al*., 2000). Knox *et al*. (2003) proposed that such highly motile spermatozoa may fertilise oocytes during IVF or be selected for insemination in ART procedures such as ICSI. Asymptomatic seminal ureaplasma infection may actually confer a positive selectivity for this microorganism in such procedures (Knox *et al*., 2003). *Ureaplasma* spp. infections can also result in tissue damage, through production of membrane-damaging hydrogen peroxide and superoxide (Meier and Habermehl, 1990). Such reactive oxygen species may inhibit motility and the acrosome reaction of spermatozoa (Aitken, 1995). Despite these mechanisms, inconsistent results have been reported when performing semen (andrology) analysis on ureaplasma-infected samples, as per the World Health Organisation criteria (World Health Organisation, 1999). In some studies andrology variables have been similar for ureaplasma culture positive and negative semen samples (Cintron *et al.*, 1981). Conversely, others have noted reduced numbers of spermatozoa (Upadhyaya *et al.*, 1984), reduced motility

(Naessens *et al.*, 1986) and morphological alterations of spermatozoa infected or co-incubated with ureaplasmas (Nunez-Calonge *et al.*, 1998).

2.7.1b Mycoplasma spp

Several independent studies have also shown that *Mycoplasma* spp. can attach to spermatozoa and remain adherent to spermatozoa after standard ART washing procedures (Hill *et al.*, 1987, Keith *et al.*, 1984, Knox *et al.*, 2003). Spermatozoa pre-incubated with *M. hominis* demonstrated lower rates of egg penetration *in vitro* (Busolo and Zanchetta, 1985, Fowlkes *et al.*, 1975). However, despite binding to spermatozoa, an infection by *M. hominis* is frequently associated with female, rather than male infertility (Taylor and Frydman, 1996). Knox *et al.* (2003) reported adverse ART pregnancy outcomes when standard ART washing procedures did not remove *M. hominis* from spermatozoa.

Another mycoplasma, *M. genitalium* is flask shaped and possesses a specialised central 'tip,' which adheres to host cells but also enhances spermatozoa motility (Taylor, 2005). *M. genitalium* is also able to attach to the head, midpiece and tails of spermatozoa (Svenstrup *et al.*, 2003), and to cells lining the lumen of the uterine tubes (Baseman *et al.*, 1996). Attachment to spermatozoa and genital tract epithelia can result in inflammation and infertility.

Whilst *M. hominis* is also proposed to inhibit spermatozoa-egg penetration by binding to glycolipid receptors on the cell membrane of spermatozoa (Busolo and Zanchetta, 1985), *M. genitalium* is able to attach to the head, midpiece and tail of

spermatozoa, causing agglutination of spermatozoa and thus impairment of motility (Svenstrup *et al.*, 2003).

These reports support a role for the genital mycoplasmas in adverse IVF pregnancy outcomes particularly if they are present on the surface of spermatozoa at the time of fertilisation. However, further investigations of the presence of ureaplasmas and genital mycoplasmas within semen, washed semen and UGT clinical specimens are needed to confirm these hypotheses.

2.7.2 *Chlamydia trachomatis*

The ability of *C. trachomatis* to attach to spermatozoa has been confirmed using immunofluorescence and transmission electron microscopy (Friberg *et al.*, 1987, Wolner-Hanssen and Mardh, 1984). *In vitro* experiments demonstrated that spermatozoa incubated with elementary bodies of *C. trachomatis* (serovars D, H, I and C) had decreased motility after 60 minutes incubation but retained the ability to progress forward (Wolner-Hanssen and Mardh, 1984). However, live spermatozoa with adherent *C. trachomatis* have been recovered from the peritoneal cavity of women with salpingitis, which supports the proposal that regardless of the motility or morphology, chlamydia-infected spermatozoa are able to travel to the UGT (Friberg *et al.*, 1987). Furthermore, Vigil *et al*. (2002 a) observed *C. trachomatis* elementary bodies attached to the surface of spermatozoa, using scanning electron microscopy and transmission electron microscopy, within chlamydia-infected spermatozoa collected from infertile men. The presence of C. *trachomatis* within semen collected from these men was not associated with decreased spermatozoal motility.

More recent studies have co-incubated human spermatozoa and either *C. trachomatis* or *C. trachomatis* (serovar E) lipopolysaccharide. These studies demonstrated that the lipopolysaccharide component of the Gram-negative cell wall was spermicidal (Hosseinzadeh *et al.*, 2001), suggesting that spermatozoa may be killed by chlamydial infection resulting in reduced viable numbers in seminal fluid and reduced fertility.

2.7.3 *N. gonorrhoeae*

Another bacterial species that can attach to spermatozoa and affect fertility is *N. gonorrhoeae* (Gomez *et al.*, 1979). Even spermatozoa immobilised by this species may be transported to the female UGT and adversely affect pregnancy outcomes. Furthermore, the spermatozoa motility may be decreased as a result of the twisting structure created around the tail by attachment of gonococcal pili (Howard, 1971).

2.7.4 *E. coli*

E. coli can also adhere to the surface of human spermatozoa and the attachment is facilitated by the interaction of the bacterial pili and the plasma membrane of the spermatozoa (Sanchez *et al.*, 1989). Motility studies conducted by Diemer *et al.* (1996) showed that *E. coli* attached to spermatozoa almost immediately during co-incubation, rapidly immobilising the spermatozoa. However, spermatozoa with adherent *E. coli* are unlikely to remain present in the washed semen fraction after IVF semen processing protocols, and therefore are unlikely to affect adversely IVF treatment outcomes.

E. coli is the most prevalent microorganism isolated from community acquired, ascending urinary tract infections (Gould, 2010). The motility of *E. coli* and its propensity for attachment to epithelial cells (Fowler and Zabin, 1977, Thomas *et al.*, 1982) enhances the ability of this bacterium to ascend through the genitourinary tract and into the UGT of both women and men.

Whilst according to the literature, only five bacteria reportedly are able to attach to spermatozoa, there is evidence that uterine contractions during intercourse, menstruation and parturition may transport spermatozoa with adherent microorganisms into the upper genital tract (Drobnis and Overstreet, 1992). This is the case for women trying to achieve both natural and ART pregnancies. Further investigations are required to determine the potential of microorganisms to 'hitch-hike' to the female UGT, in the absence of motile spermatozoa, and to determine the prevalence of species within semen that are able to access the UGT tissues.

2.7.5 Sperm DNA fragmentation and apoptosis

Recently, sperm DNA fragmentation has been used as a measure of apoptosis and sperm quality. Many microbial species reportedly cause DNA fragmentation including those that can attach to spermatozoa: *C. trachomatis*, *Mycoplasma* spp. (Gallegos *et al.*, 2008), enteric Gram-negative bacteria (*Salmonella* spp., *E. coli*, *Pasteurella* spp.) (Gorga *et al.*, 2001), *S. aureus* (Villegas *et al.*, 2005 a, b) and the yeast *Candida albicans* (Burrello *et al.*, 2004). Species that can attach to spermatozoa can gain access to the female upper genital tract or zygote and may affect IVF pregnancy outcomes by damaging the DNA of the sperm. The impact of DNA fragmentation in spermatozoa (used for IVF or ART insemination) remains to

be fully elucidated, however in some studies, DNA fragmentation was associated with reduced: fertilisation rates (Benchaib *et al.*, 2003), embryo quality, and development (Borini *et al.*, 2006) and pregnancy rates (Henkel *et al.*, 2004).

2.8 Assisted reproduction and upper genital tract infections

In a review of trans-vaginal oocyte retrieval-related tubo-ovarian abscesses (Medline search in English language until May 2005), Sharpe *et al.* (2006) found only 39 reports of pelvic abscess complications associated with this procedure. Of these cases, only seven occurred with a concurrent pregnancy. Numerous organisms, including *Streptococcus* group B and D, *S. viridans, E. coli, Bacteroides* spp., *Peptostreptococcus* spp., *Enterococcus* spp., *S. aureus, Atopobium vaginae, Citrobacter* spp. and *Klebsiella* spp., were implicated as the causative factors of these infections. However, this review failed to report whether any of these 39 women had prior ART treatment that included a trans-vaginal oocyte retrieval procedure. This is an important omission, as the retrieval procedure in itself provides an opportunity for normal regional flora of the LGT to be introduced into the UGT. Alternatively, ascending invasive infections by opportunistic normal regional flora or pathogenic microorganisms within the LGT may be the source/cause of tubo-ovarian abscesses.

Trans-vaginal oocyte retrieval may also reactivate a persistent asymptomatic UGT infection, potentially resulting in tubal occlusion; however, as the role of microorganisms and chronic underlying infection in adverse ART outcomes has been largely ignored and insufficiently investigated, it is likely to be under-reported.

A study of 33 follicular fluid specimens collected from women undergoing IVF cycles, (Knox, unpublished data) detected one or more microorganisms in 68.8% of the samples. Microorganisms detected in follicular fluid included: *Actinomyces* spp., *Bacillus* spp., *Clostridium* spp., *Enterococcus faecalis*, *G. vaginalis*, *Lactobacillus* spp., *Mobiluncus* spp., *M. curtisii*, *Peptostreptococcus* spp., *Propionibacterium* spp., *S. epidermidis*, *Streptococcus* spp. and *C. trachomatis*. In retrospective analyses, women with infected/colonised follicular fluid had decreased fertilisation rates after ICSI (66.7%) and IVF (70.8%), compared with those rates reported in uninfected/non-colonised women (87.5% and 77% respectively). Prior trans-vaginal oocyte retrieval procedures were associated with lower fresh embryo transfer rates in patients both with infected and uninfected follicular fluid; however, the sample size was not large enough for the results to be statistically significant. It is also possible that trans-vaginal oocyte retrieval reactivated normal regional flora inoculated into the UGT at the time of previous procedures and caused adverse ART outcomes in subsequent cycles.

2.9 Antimicrobial therapy

Antimicrobial treatment of males that had infected semen demonstrated the presence of 'new' bacterial species within semen, that is, bacteria that were not detected in the original infected semen of male partners who had positive semen cultures at the time of semen andrology screening at the beginning of ART treatment (Huyser *et al.*, 1991, Liversedge *et al.*, 1996). The 'new' species were detected within the semen two weeks after antimicrobial treatment and it is, therefore, possible that antimicrobial treatment is selective for microorganisms, allowing species resistant to the specific antimicrobial to overgrow the normal regional flora in the genital tract

for possible transmission to the female partner (Huyser *et al.*, 1991, Liversedge *et al.*, 1996). Similarly, overgrowth of pathogens in the female LGT after antimicrobial treatment may also result in transmission of 'resistant' bacterial strains to the male partner, or the male partner may be re-infected with bacteria that were previously eradicated (Huyser *et al.*, 1991, Liversedge *et al.*, 1996).

Couples in which the male partner had bacteria, including enterococci, Gram-negative bacilli, streptococci, staphylococci, diphtheroids or the yeast *Candida albicans*, in his semen (asymptomatic colonisation), who were subsequently treated with antimicrobial agents, experienced similar ART fertilisation rates to couples with bacteria-free semen. However, the female partner of couples where the asymptomatic male partner was treated with antimicrobials demonstrated significantly increased numbers of Gram-negative bacteria in the high vaginal swab (Liversedge *et al.*, 1996). Antimicrobials routinely used to treat prostatic infections (nitrofurans, aminoglycosides, macrolides and sulphur drugs) may have an adverse affect on sperm function. These antimicrobials concentrate within seminal fluid, which is then deposited into the vagina at the time of sexual intercourse. Primate models have been used to demonstrate adverse alterations in normal vaginal flora from lactobacillus–dominant to enteric bacteria following a single vaginally administered dose of amoxicillin (Winberg *et al.*, 1993). Taken together, these observations offer a sound rationale for not prescribing prophylactic antimicrobial treatment to uninfected couples with asymptomatic LGT colonisation whilst participating in ART treatment.

In a small study assessing DNA fragmentation of sperm caused by infection of *C. trachomatis* or genital mycoplasmas, antibiotic therapy was successful in improving

spermatozoa morphology and in decreasing spermatozoa DNA fragmentation. This resulted in increased pregnancy rates for couples conceiving in the period of three to six months following antimicrobial treatment of infected males, but not for those couples who tried to conceive during the antimicrobial treatment period (Gallegos *et al.*, 2008).

Microbial contamination of the embryo culture system has been reported following antimicrobial treatment of the male partner and this, again, was associated with a decrease in the lactobacillus-dominant vaginal flora in the female partner (Liversedge *et al.*, 1996). Screening of both partners prior to commencement of ART cycles may provide valuable information regarding LGT colonisation of the vagina in females and of the distal urethra in males. The identification of single species of pathogenic bacteria in the female or male LGT potentially may lead to targeted, narrow spectrum antimicrobial therapy for couples prior to ART treatment, and such treatment may be less likely to have detrimental effects on the vaginal normal regional flora or to promote emergence and persistence of resistant pathogenic species.

2.10 Biofilms

2.10.1 Common structural features of biofilms

A biofilm is an aggregate of microorganisms that confers protection and enhances survival of bacteria within the biofilm environment. In nature, over 99% of bacteria exist in heterogeneous communities. Biofilms represent a complex multi-cellular microbial population regulated by quorum sensing, cell-cell signalling and the secretion of signalling molecules (Patterson *et al.*, 2009). Cells within various

regions of the biofilm exhibit different patterns of gene expression, which enhances their ability to resist host immune defences and antimicrobial therapy. Mature biofilms exhibit a variety of phenotypes due to the modifications in their three-dimensional structure, which occur in response to changes in cell density, osmolarity, temperature, pH and nutrient supply (Fux *et al.*, 2005 a). Bacterial metabolic by-products, including enzymatic virulence factors, which may have synergistic or antagonistic effects on other members of the surrounding microbial population, are also found in mature biofilms. These factors may adversely affect a host, which supports the growth of a biofilm, resulting in pathology and a diminished immune response that fails to destroy bacteria within the biofilm. However, the biofilm may cause inflammatory damage to the surrounding tissues (Cochrane, 1988).

Biofilm formation occurs in a similar manner irrespective of the site — human or environmental (Patterson *et al.*, 2009). For a biofilm to form there must first be a substratum, a solid surface in contact with a liquid. A conditioning film comprised of macromolecules (polysaccharides and proteins) in the liquid then coats the substratum; it forms the first anchoring point for the planktonic (free-floating) bacterial cells. The bacteria first attach in a reversible fashion to the conditioning film, and then increase their numbers so that the population develops stronger bonds and becomes permanent. During this consolidation phase, bacteria produce exopolysaccharide or slime, and upward growing microcolonies begin to appear. As the biofilm matures over time, the extracellular matrix remains in contact with the liquid. This interface is protective, minimising the effect of host immune defences and the penetrability of many antimicrobials or chemical treatments (Patterson *et al.*, 2009). Biofilms may be more resistant to antimicrobials because of: (1) the inability

of the antimicrobial to penetrate the full depth of the biofilm (Stewart *et al.*, 1998); (2) the presence of slow-growing or metabolically-inactive cells (due to nutrient depletion, a viable but non-cultivable state) that are resistant to some antimicrobials (Rayner *et al.*, 1998); and (3) sessile rather than planktonic growth of cells in the biofilm (Costerton, 1999, Costerton *et al.*, 1999). As the liquid surrounding the biofilm is not static, shearing forces can relocate some microorganisms from the biofilm to new sites where this process can begin again.

2.10.2 Biofilm formation and persistence within the genital tract

Many of the bacteria present within follicular fluid can also be isolated from the female LGT and have been shown to form biofilms: *in vitro*, when cultured on vaginal epithelial cells (Patterson *et al*., 2009); *in vivo* on intrauterine devices (Pruthi *et al.*, 2003); and in amniotic fluid sludge aspirated from women with intrauterine infection (Romero *et al.*, 2008). The study of *G. vaginalis* biofilms within the genital tract has aided our understanding of the persistence of some genital tract infections. Previously, investigations of *G. vaginalis* biofilm formation (over a period of 72 hours) were used to model bacterial vaginosis; however, these experiments included only this species in pure culture and not a polymicrobial biofilm characteristic of this condition (Patterson *et al*., 2009). The ability of *G. vaginalis* to form biofilms may be a reason for the failure of antimicrobial therapy for the treatment of infections caused by this bacterium. Swidinski *et al*. (2005) found that women diagnosed with bacterial vaginosis demonstrated a persistent polymicrobial vaginal biofilm for up to five weeks post-treatment. However, *in vitro* experiments have demonstrated that *Lactobacillus* spp. can interrupt a well-established *G. vaginalis* biofilm, kill bacterial cells and establish growth within the spaces of the biofilm (Saunders *et al.*, 2007).

Modern molecular techniques have demonstrated that biopsies of biofilms from women with bacterial vaginosis are polymicrobial, suggesting that *G. vaginalis* within a biofilm creates an environment where other bacteria can flourish in high numbers, but remain undetectable by conventional culture techniques (Swidinski *et al*., 2005). It has also been reported that opportunistic or sexually transmitted pathogens can co-colonise with *Lactobacillus* spp. on urogenital tract cells; but after lactobacilli become established, they can cause pathogens to detach from the epithelium, which may also be a defence mechanism (Spurbeck and Arvidson, 2008, Vielfort *et al.*, 2008). Despite previous research, the individual microorganisms within the normal regional flora microbial biofilms within the vagina are yet to be fully characterised, largely due to the inadequate current routine screening methods (culture techniques rather than molecular techniques) used to test these specimens (Witkin *et al*., 2007).

Biofilms offer a persistent form of microbial growth. The presence of metabolically dormant microorganisms within a biofilm phenotype in the female UGT may be another cause of infertility, warranting further investigation.

2.11 Viruses capable of causing upper genital tract infections

2.11.1 Exogenous viruses capable of causing genital tract infection

Couples entering ART treatment cycles in Australia currently are screened for sexually transmitted viruses – HIV and HBV and HCV viruses (Kovacs and Allan, personal communication). This review highlights that there are many viruses capable of infecting the reproductive tissues and fluids. The human exogenous retroviruses are HIV and human T-cell lymphotropic virus (HTLV) (Bezold *et al*., 2007, Bertrand

et al., 2004, Devaux *et al*., 2003, Quayle *et al*., 1998). Whilst the role of HIV as an STI is well established, its ability to infect gametes remains controversial. Other viruses reported in genital tract infections are the herpes viruses (including Epstein-Barr virus, CMV, and herpes simplex virus) (El Borai *et al*., 1998, Kapranos *et al*., 2003) and the HPV viruses (Spandorfer *et al*., 2006), which are currently not screened for in couples entering ART treatment cycles.

The presence of multiple sexually transmissible viruses within the genital tracts of infertile couples highlights the need for screening both partners prior to treatment, followed by the initiation of antiviral therapy where indicated and the re-evaluation of fertility following resolution of these infections. There is normally no clinical follow up for individuals after treatment for HPV infection; however, these infections are known to be latent and thus may be reactivated later. Viral co-infection adds further complexity to the problem of infertility since it has been associated with enhanced virus infectivity and an increased risk of transmission to a partner and/or offspring (Toth *et al.*, 1997). Further investigations of all viruses and their ability to infect the male and female UGT, and their effects on ART outcomes will increase our knowledge of the association of viral infections, with infertility and adverse ART outcomes; however, screening for the presence of viruses was beyond the scope of this current project.

2.12 The ovary

2.12.1 Function of the ovary

The function of the ovaries is to produce: (1) gametes (oocytes); and (2) the hormones involved in the regulation of the ovarian (menstrual) cycle, oestradiol,

progesterone, inhibin and relaxin and those involved in maintaining early pregnancy (progesterone) (Saladin, 2007).

2.12.2 The menstrual cycle and steroid hormones

There are two phases of the menstrual cycle: the follicular phase in which oocytes mature in preparation for ovulation; and the luteal phase, which occurs directly after ovulation and is the phase preceding menstruation when fertilisation and implantation may occur. In the follicular phase, the independent growth of the primordial follicle is enhanced in response to follicle stimulating hormone (FSH) (Speroff and Fritz, 2005).

FSH is converted to oestradiol and it is these two hormones, which stimulate proliferation of the granulosa cells. Oestradiol levels within the antral (dominant) follicle continue to increase until oestradiol levels are sufficient to maintain the elevated peripheral concentrations necessary to induce a surge of luteinizing hormone (LH) to drive the cycle to ovulation (Figure 2.1). LH initiates progesterone production and luteinization within the granulosa cells (Speroff and Fritz, 2005). Ovulation follows the LH surge in response to the progesterone induced proteolytic enzymatic activity and prostaglandins, leading to the rupture of the ovarian follicle wall (Figure 2.2). In the early luteal phase, new vasculature forms within or surrounding a follicle due to secretion of the growth factor, vascular endothelial growth factor (VEGF). Progesterone and oestradiol act to suppress gonadotropins and the development of alternative follicles. In the absence of pregnancy (and human chorionic gonadotropin (hCG) secreted from the foetus) the corpus luteum regresses, causing a decrease in oestradiol and progesterone and a rapid increase in

gonadotropin releasing hormone. Menstruation follows and the cycle begins again with an increase in FSH to rescue the primordial follicles and increase the follicular fluid volume within an antral follicle (Speroff and Fritz, 2005).

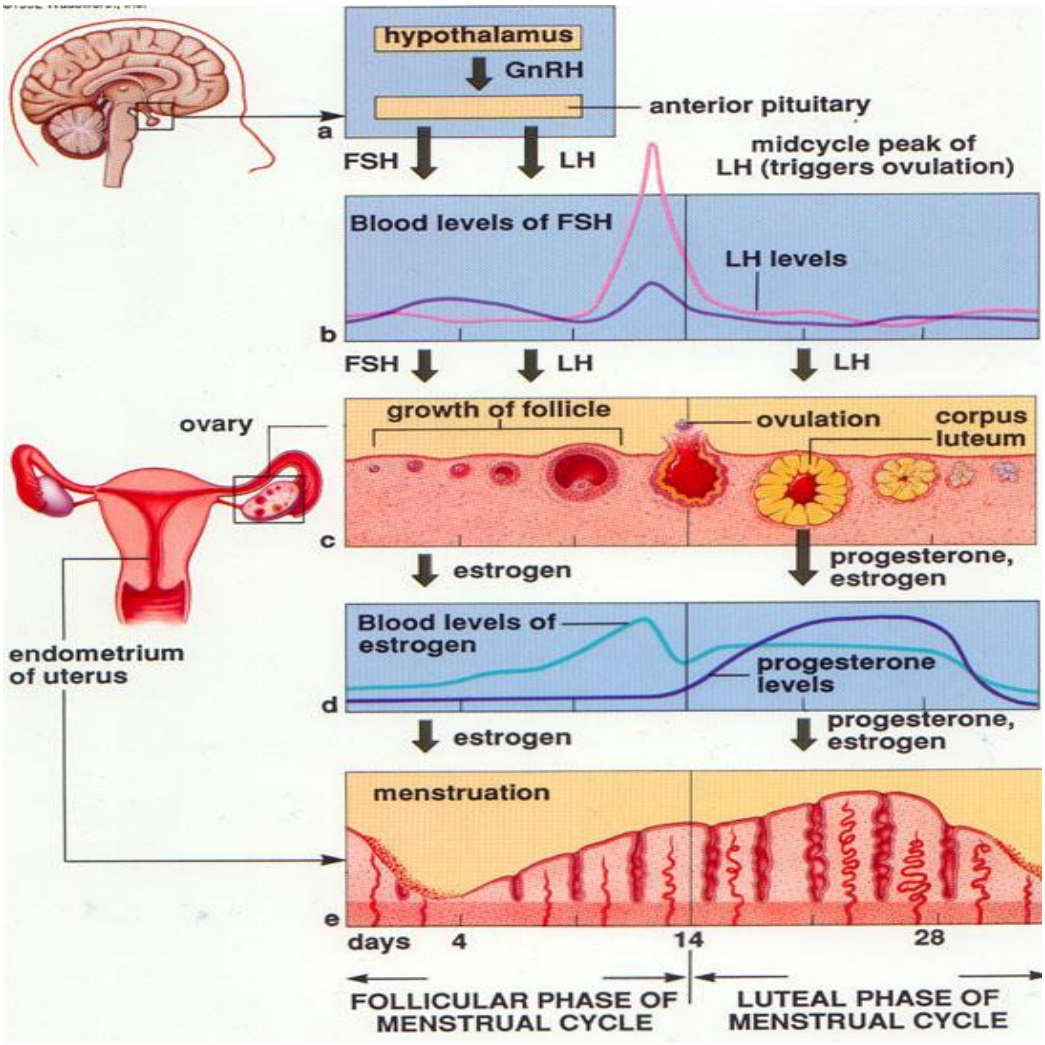


Figure 2.1 The menstrual cycle (soc.ucsb.edu). Changes in the steroid hormone levels of oestradiol and progesterone throughout the menstrual cycle. FSH levels cause growth and maturation of the dominant follicle. The LH surge triggers ovulation of the mature oocyte in the second half of the menstrual cycle.

2.12.3 Follicular fluid within ovarian follicles

Follicular fluid is a hypocoagulable, semi-viscous fluid containing proteins, inorganic compounds, carbohydrates, mucopolysaccharides, lipids, gonadotropins, steroid hormones, immunoglobulins, cytokines, complement components and growth factors (Edwards, 1974, Shimada *et al.*, 2001). Most proteins in follicular fluid are plasma proteins, which migrate through the semi-permeable follicular epithelium or are locally secreted products of the granulosa or thecal cells (Edwards, 1974). However, there are numerous differences between the concentrations of these elements in plasma compared to the concentration in follicular fluid, particularly in the proteins involved in the hormonal control of the menstrual cycle (Shimada *et al.*, 2001). The porosity of the follicle membrane is modulated by hormones and is increased at the time of ovulation. The oocyte is expelled from the follicle by a gentle flow of follicular fluid and 'anchored' to the exterior of the ovary, from where it is then brushed into the ampulla of the uterine tube by the fimbriae (Edwards, 1974).

Follicular fluid is potentially an excellent growth media, which may support microbial growth. The extensive vasculature within the ovary and in individual follicles ensures that the supply of nutrients is optimal and that waste removal is efficient.

Alterations in the coagulability of follicular fluid have been observed in infected follicular fluid specimens that have formed a fibrin clot (Knox, unpublished data). It was proposed that this was due to the presence of bacteria found in > 60% of follicular fluids tested in the preliminary study. Thus, future investigations should

aim to determine, which follicular fluid components are altered by the presence of microorganisms or which microorganism components induce coagulation. An interesting finding in the literature is that follicular fluid is hypocoagulable and this highlights the clinical importance of clotting in follicular fluid specimens (Edwards, 1974). It may be that the formation of a clot is an indicator of infection/abnormal proteins.

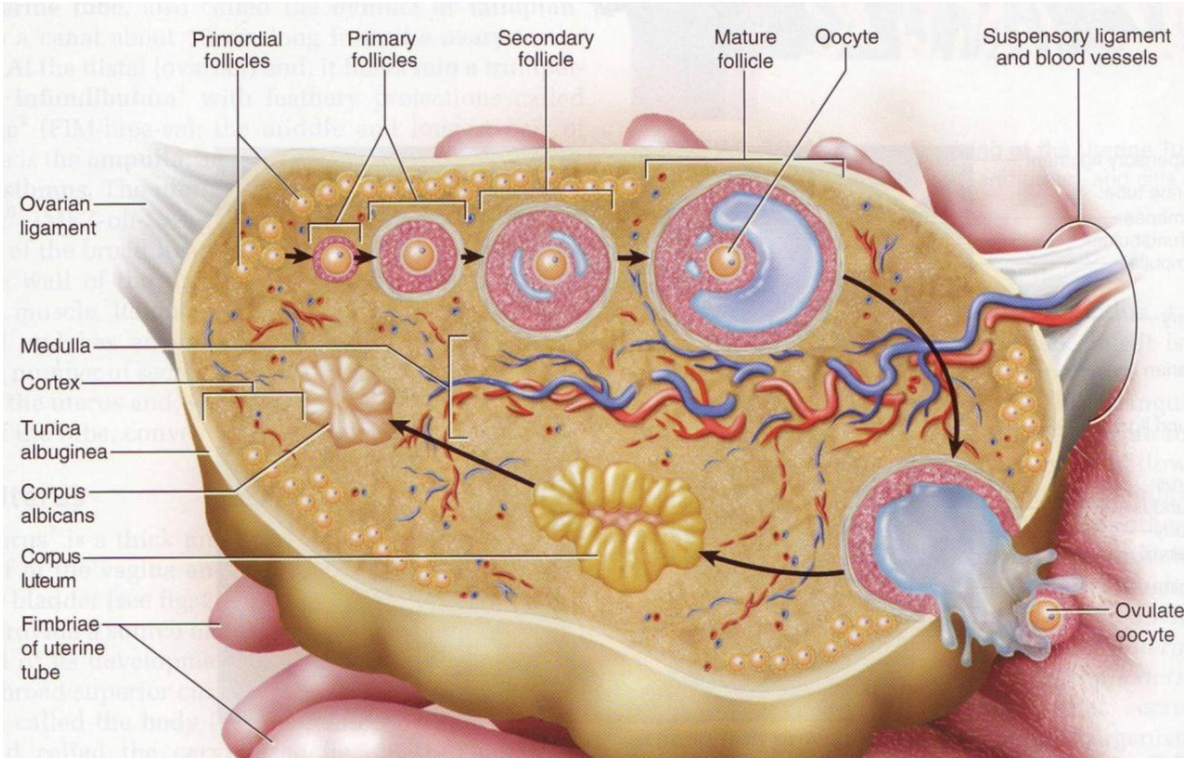


Figure 2.2 Transverse section of ovarian follicle maturation (Saladin, 2007). Schematic representation of the *in vivo* maturation of a dominant ovarian follicle and the increase in follicular fluid volume, until rupture at the time of ovulation.

The clotting system is responsible for minimising blood loss, and modulating the immune system through the release of inflammatory mediators, which induce clot formation to limit microbial dissemination (Yun *et al*., 2009). Thrombin is the enzyme critical to generating coagulation (Bungay *et al*., 2006), however, within the ovary, thrombin also functions as a potent cell-signalling molecule (Roach *et al*.,

2002). The concentration of thrombin generating proteins produced in the follicular fluid is lower than that produced in the blood plasma, which results in a maximum thrombin concentration within the follicular fluid of only 0.40 nM / L compared to 174.16 nM / L within plasma (Bungay *et al*., 2006). The level of thrombin produced within the follicular fluid is frequently below the threshold (10–30 nM / L) required for clot formation (Mann *et al*., 2003). The low level of thrombin in follicular fluid was proposed as a reason for the increased *in vitro* fibrin clot time (Roach *et al*., 2001). Based on this data, follicular fluid is classified as a hypocoagulable fluid. Previous research proposed that thrombin regulation of cell signalling molecules including cytokines, growth factors and proteolytic enzymes were essential for normal folliculogenesis and ovulation (Hirota *et al*., 2003). Therefore, altered levels of cell signalling molecules such as cytokines, present in response to the host immune response to microorganisms, may result in increased thrombin levels and clot formation.

Tissue factor, the key mediator of thrombin generation is synthesised and expressed by the granulosa cells of the ovarian follicle (Salmassi *et al*., 2001). Several cytokines including IL-6 reportedly enhance the expression of tissue factor within the granulosa cells and IL-6 is also a pro-inflammatory cytokine, up regulated by the presence of microorganisms. The enhanced expression of IL-6 within some follicular fluids may be a result of the presence of some microbial species within follicular fluid and this may lead to an increase in tissue factor activity, which in turn would cause the abnormal clot formation observed in some of the follicular fluids containing microorganisms that were collected from IVF patients. In addition to activating the host immune response, some microorganisms express virulence

factors, including bacterial omptins, which can both enhance and inhibit the clotting cascade (Yun *et al*., 2009). The formation of a clot within follicular fluids containing microorganisms is a rare observation; that may reflect variations in the host immune response (cytokine expression) and/or the production of microbial virulence factors for example, omptins, which are capable of altering the coagulation cascade.

During an IVF treatment cycle, administration of exogenous steroid hormones triggers the simultaneous maturation of multiple ovarian follicles. The reported levels of steroid hormones within the follicular fluid of these women undergoing IVF treatment far exceeds those found in women with normal menstrual cycles: levels are approximately eight fold higher for oestradiol and three fold higher for progesterone (Kushnir *et al.*, 2009).

Furthermore, bacteria utilise steroid hormones as growth factors, and this may provide a selective advantage in this anatomical niche (Kornman and Loesche, 1982). The susceptibility of the female reproductive tract to infection is also modulated by the steroid hormone concentrations of oestradiol and progesterone (Sonnex, 1998). The diagnosis of female genital tract infections can be directly related to the stage of the menstrual cycle. For example, elevated levels of oestrogens found in pregnant women have been associated with an increased incidence of candidiasis (Fidel *et al.*, 1996). Chlamydial infection is more frequently diagnosed just prior to menstruation, and gonorrhoea is commonly diagnosed at the time of menstruation when both progesterone and oestradiol levels are low (Sweet *et al.*, 1986). Bacterial vaginosis (Furr, 1991, Sautter and Brown, 1980) and infection with genital mycoplasmas (Furr and Taylor-Robinson, 1993, Taylor-Robinson and Furr,

1993) have also been more frequently reported in the first half of the menstrual cycle when oestradiol levels peak.

Oral contraceptives also alter the steroid hormone concentrations within the female genital tract throughout the menstrual cycle. Women may be prescribed either combined oral contraceptives or progesterone only alternatives. Research suggests that steroid hormones influence the local immune response within the genital tract (Brabin *et al.*, 2005 a, b) and thus the susceptibility to genital tract infection (McGregor *et al.*, 1990). Another study, however, has not reported the significant associations between oral contraceptive use and genital tract infection (Ness *et al.*, 2001), which is possibly due to the decreased concentrations of oestradiol and progesterone included in modern contraceptives. It is more likely that the advantages and disadvantages of oral contraceptives are dose and species dependent, such that the effect of oral contraceptives on the genital tract microflora is due to the type and does of steroid hormone, and the specific bacterial species present within the follicular fluid. Therefore, it can also be proposed that the steroid hormone concentrations in follicular fluid may influence the growth and survival of microorganisms in follicular fluid and other genital tract sites.

2.12.4 Asymmetrical Innervation and Vascularisation of the Ovaries

The ovaries demonstrate morphological, biochemical, physiological and pathological asymmetry (Gerendai and Halasz, 1997). There is a predominance of right-sided brain structures (hypothalamo-pituitary system) controlling ovarian function, which results in greater supraspinal innervation of the left ovary (Toth *et al.*, 2007). In contrast, the right ovary has a greater supply by sympathetic afferent fibres (Klein

and Burden, 1988). The vascularisation of the ovaries is also asymmetrical. The ovaries have dual arterial and venous blood supply with the arterial supply derived from the ovarian (gonadal) arteries and from the adnexal branches of the uterine artery. The venous system parallels the arterial supply, with the exception that the left ovarian vein drains into the left renal vein, and the right ovarian vein drains into the inferior vena cava (Mittwoch, 1975).

Since the left and right ovaries are separately vascularised, they function independently, where maturation of a single follicle and subsequently ovulation can occur in either ovary in any given cycle. This independence in function enables the preservation of fertility in cases of unilateral ovarian damage, removal or dysfunction. Since the ovaries are independently vascularised, it is possible that there are differences not only in pathology but also in the microflora of each ovary.

2.13 Cytokines

The presence of microorganisms within the UGT results in an immune response mounted by the host. If there are microorganisms present in follicular fluid in the ovary, then it is likely that the host immune response will be enhanced at this site.

2.13.1 Cytokines and reproduction

Expression of cytokines appears to play an essential role in reproduction − including ovarian function, ovarian steroidal secretion, corpus luteum function, embryonic development and implantation (Vujisic *et al.*, 2006). Cells of the white cell lineage constitute a major component of the ovarian interstitial compartment, and play an important role in the regulation of the reproductive system through the secretion of

growth factors and cytokines. White blood cells undergo cyclical changes throughout the menstrual cycle: mast cell degranulation and histamine release facilitate increased blood flow at ovulation; whereas eosinophils and T lymphocytes signal activated monocytes and macrophages to the corpus luteum for luteolysis (Adashi, 1989). Macrophages have many functions within the female reproductive tract and activation of macrophages occurs during an immune response to pathogens. These immune effectors also play a significant role in homeostasis for reproductive function via cytokine production and secretion (Table 2.3) (Gordon and Rowsey, 1999). Tissue remodelling and repair occurs throughout the menstrual cycle as an essential process to control endometrial cycling, preparation for implantation and ongoing pregnancy (Wu *et al.*, 2004). This remodelling is facilitated by macrophages.

2.13.2 Cytokines and STIs

The long-term sequelae associated with sexually transmitted diseases caused by pathogens including *C. trachomatis*, *N. gonorrhoeae* and *T. pallidum* are due in part to the up-regulation of pro-inflammatory cytokines. The host immune response increases the expression of many immune effectors in an attempt to clear, and resolve STIs (Table 2.4).

Table 2.3 The role of cytokines in female reproductive tract function

Cytokine	Role in female reproductive tract	Study
GMCSF[1]	Regulator and activator of granulocytes and macrophages	
Gro α[2]	Neutrophil chemoattractant	Sager *et al.*, 1992
IL-1[3]	Ovulation promotion Induces IL-6 production for angiogenesis Enhances IL-8 production Inhibited by progesterone Inhibited by oestradiol Induces NO production	Brannstrom *et al.*, 1993, Mori *et al.*, 1990 Hu *et al.*, 1988, Polan *et al.*, 1989
IL-10	Inhibition of progesterone production Anti-inflammatroy antagonist to IL-1, IL-2, IL-6, TNF α	Geva *et al.*, 1997
IL-1 ra	Antagonises IL-1 to prevent tissue damage after ovulation	Buscher *et al.*, 1999
IL-1 α	Stimulates T and B lymphocyte proliferation Stimulates prostaglandin production	Ribardo *et al.*, 2001 Ribardo *et al.*, 2001
IL-1 β	Inhibits apoptosis by NO production Stimulates T and B lymphocyte proliferation Stimulates prostaglandin production	Chun *et al.*, 1995 Ribardo *et al.*, 2001 Ribardo *et al.*, 2001
IL-2	Progesterone stimulation HCG suppression	Wang *et al.*, 1991
IL-6	Role in steroid hormone production Reduces granulosa cell proliferation Inhibited by oestradiol	Alpizar and Spicer, 1994 Bukulmez and Arici, 2000 Hu *et al.*, 1988
IL-8	Angiogenesis Prevention of infection Prevention of tissue damage after ovulation	Koch *et al.*, 1992 Buscher *et al.*, 1999 Buscher *et al.*, 1999
LIF[4]	Oestradiol biosynthesis	Bukulmez and Arici, 2000
MCP-1[5]	Monocyte attraction and activation	
TNF α[6]	Androgen production and induction of LH receptors Inhibited by oestradiol	Norman and Brannstrom, 1996 Hu *et al.*, 1988

[1]Granulocyte macrophage colony stimulating factor (GMCSF), [2]Growth regulated oncogene (Gro) α, [3]Interleukin (IL), [4]Leukaemia inhibitory factor (LIF), [5]Monocyte chemoattractant protein (MCP)-1, [6]Tumour necrosis factor (TNF).

Table 2.4 Cytokines up regulated by STIs

Microorganism	Cytokines up regulated by infection	References
C. trachomatis	IL-1, IL-6, IL-10, IL-8, IL-17, IL-22 IFN[1] γ, TNF α GMCSF	Ohman *et al*., 2011, Jha *et al*., 2011, Rasmussen *et al*., 1997, Buchholz and Stephens, 2006
Genital mycoplasmas	IL-1 α, IL-1 β, IL-6, IL-8, GCSF[2], GMCSF, MCP-1	Ryckman *et al*., 2008, McGowin *et al*., 2009
Hepatitis virus	IL-6, IL-17, IL-22, IL-23	Pan *et al*., 2011, Xu *et al*., 2011
HSV	IL-6, IFN α, IFN γ	Cunningham *et al*., 2006, Tang and Rosenthal, 2010
HIV	IL-1 β , IL-6, IL-8, IL-12, IFN γ	Lee *et al*., 2007, Cota *et al*., 2000, Kaul *et al*., 2008
HPV	IL-12p40, IL-15, IFN γ, TNF α	Stanley, 2001, Chan *et al*., 2011
N. gonorrhoeae	IL-6, IL-8, IL-17, IL-23, TNF α	Gagliardi *et al*., 2011, Wesler *et al*., 2005
T. pallidum	IL-1 β , IL-6, IL-10, TNF α	Liu *et al*., 2010, Knudsen *et al*., 2010
T. vaginalis	IL-1 β, IL-8, TNF α	Shaio *et al*., 1995, Cauci and Culhane, 2007, Simhan *et al*., 2007

[1]Interferon (IFN), [2]Granulocyte colony stimulating factor (GCSF).

2.13.3 Cytokine roles and subsets

Cytokines are polypeptides and glycoproteins, produced by cells as signalling compounds following activation of the innate or adaptive immune response (Husband *et al.*, 1999). They are particularly important in innate and adaptive immune responses including inflammation. CD4+ T helper cells are the major immune cells involved in cytokine production, and cytokines have been isolated from resident and infiltrating leukocytes in follicular fluid; ovarian somatic cells; and

from endometrial cells. Cytokines are produced by activated T cells, which are induced by cytokines and growth factors, each expressing different chemokine receptors (King *et al*., 2008) (Figure 2.3).

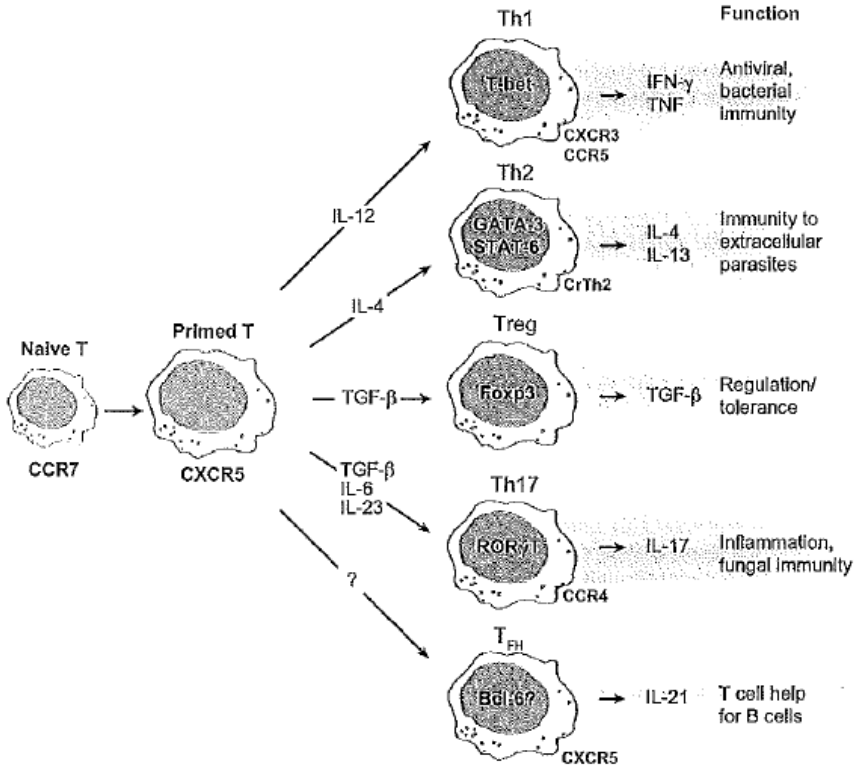


Figure 2.3 Effector T-cell differentiations (King *et al*., 2008). A schematic diagram of the effector T-cell differentiation that occurs as naive T-cells are primed in response to physiological cues or threats. Cytokines and growth factors cause further differentiation of primed T-cells into effector T-cells, which are capable of producing a specific suite of cytokines in response to stimuli, aimed at returning homeostasis. Each T-cell subset has specific cytokine receptors for a controlled immune response.

1) The first group of cytokines are produced by:

a) T helper (Th) 0 cells: these precursor cells can be converted into either Th1 or Th2 cells or are capable of their own production. Th0 cells secrete tumour necrosis factor (TNF) α and granulocyte macrophage-colony stimulating factor (GMCSF).

b) Th1 cells produce interferon gamma (IFN γ), interleukin (IL)-2 and TNF β, which are responsible for the cell mediated response.

c) Th2 cells produce IL-4, IL-5, IL-6 and IL-10, which are responsible for the antibody mediated humoral response.

d) T regulatory (Treg) cells produce tumour-like growth factor (TGF)β, responsible for immune regulation and tolerance.

e) Th17 cells produce IL-17, responsible for mediating pro-inflammatory responses.

f) T_{FH} cells produce IL-21, responsible for regulation of cell-mediated immune response via natural killer (NK) cells and cytotoxic T cell activation.

2) A second group of cytokines, the pro-inflammatory cytokines, are produced by macrophages and include IL-1, IL-6, TNF α and leukaemia inhibitory factor (LIF). These cytokines facilitate inflammation of damaged tissue and subsequent repair.

3) The third group of cytokines are the chemotactic cytokines or chemokines. This group attracts leukocytes by binding to the cell surface receptors. The chemokines are divided into four groups, based on the spacing between the first two-cysteine residues (C, CC, CXC, or CX_3C). The receptors for theses chemokines are located on the surface of leukocytes and are: CCR, CXCR, CX_3CR1 and XCR1 respectively. Receptor coupling enables activation of these immune effectors. Some chemokines

are currently under investigation for their possible role in implantation failure following IVF and in recurrent spontaneous abortion (Laird *et al.*, 2006).

2.13.4 Ovarian cytokines

Fluctuating numbers of macrophages have been detected in the ovary throughout the menstrual cycle (Brannstrom *et al.*, 1994). The role of ovarian macrophages includes regulation of follicular growth and differentiation, ovulation and corpus luteum formation and regression (Brannstrom and Norman, 1993). During follicle growth, macrophage numbers within the ovary proliferate and localise to dominant (maturing) follicles (Wu *et al.*, 2004), secreting cytokines to stimulate cellular proliferation and follicular growth, and to suppress apoptosis (Richards *et al.*, 2002). This significant increase in macrophage numbers within the ovarian theca immediately prior to ovulation results in the secretion of proteases and cytokines thought to be essential for ovulation to occur (Brannstrom *et al.*, 1995, Tadros *et al.*, 2001). The formation and regression of the corpus luteum is also modulated by macrophage-secreted cytokines related to angiogenesis (Sunderkotter *et al.*, 1994) and increased production of progesterone (Chen and Peng, 2000). Macrophages also play a significant role in ovarian dysfunction (Table 2.5); alterations in cytokine levels have been reported in follicular fluid and ovarian tissue for women with endometriosis (Fasciani *et al.*, 2000), polycystic ovary syndrome (Amato *et al.*, 2003), premature ovarian failure (Hill *et al.*, 1990) and ovarian cancer (Pisa *et al.*, 1992).

Table 2.5 Cytokines involved in ovarian reproductive pathology

Aetiology	Cytokines	Test groups	Tissue	Study
Endometriosis	IL-1 β IL-6 IL-8	Women with visually proven endometriosis	Ovarian endometriotic tissue	Fasciani *et al.*, 2000
	VEGF[1]		Follicular fluid	Van Blerkom *et al.*, 1997
Polycystic ovary syndrome	IL-6 TNF α	Hyperstimulated women[2]	Follicular fluid	Amato *et al.*, 2003
Premature ovarian failure	Class II MHC[3] positive macrophages	Women with premature ovarian failure	Ovary	Hill *et al.*, 1990
Ovarian cancer	IL-1 IL-2 IL-6 TNF α GMCSF	Ovarian cancer patients	Histologically classified ovarian tumour biopsy	Pisa *et al.*, 1992
	MCSF		Ascites fluid	Price *et al.*, 1993

[1]Vascular endothelial growth factor (VEGF), [2]Women receiving exogenous hormones for the production of multiple mature ovarian follicles for trans-vaginal oocyte retrieval and IVF, [3]Major histocompatibility complex (MHC)

2.13.5 Cytokines in assisted reproductive technology outcomes

In recent years, a number of studies have investigated the presence of cytokines in follicular fluid and correlated their presence to ART and pregnancy outcomes (see Tables 2.6 and 2.7 for a summary). In some studies, no cytokine profile has been shown to be associated with adverse ART outcomes (Asimakopoulos *et al.*, 2006, Hammadeh *et al.*, 2004); whilst there is evidence to suggest that cytokines IL-1, IL-1 α, IL-1 β, IL-12/23 (p40) and vascular endothelial growth factor (VEGF) are predictive of successful outcomes (Karagouni *et al.*, 1998, Vujisic *et al.*, 2006). By contrast, the presence of elevated levels of IL-12, VEGF and IL-15 predicted unsuccessful ART outcomes (Asimakopoulos *et al.*, 2006, Gazvani *et al.*, 2000, Vujisic *et al.*, 2006). Successful fertilisation has been associated with elevated follicular fluid levels of insulin-like growth factor binding proteins (IGFBP) (Wang *et al.*, 2006), whilst the development of good quality embryos has also been associated with these proteins within follicular fluid in addition to plasma IL-1, the IL-1 receptor (IL-1 ra) and follicular fluid IL-18 (Spandorfer *et al.*, 2003, Vujisic *et al.*, 2006, Wang *et al.*, 2006). The site-specific expression of these soluble factors highlights the significant role follicular fluid plays in controlling the outcome of early reproductive events. Adverse events, such as recurrent miscarriage, appear to represent a more systemic reaction and levels of pro-inflammatory cytokines detected within the blood plasma or endometrial tissues have been detected in women with repeated spontaneous abortion (Table 2.6) (Lim *et al.*, 2000, Shimada *et al.*, 2003).

Spandorfer *et al.* (2001) investigated abnormal vaginal flora, vaginal pro-inflammatory cytokines and idiopathic infertility in women undergoing IVF and

demonstrated a correlation between bacterial vaginosis, elevated IL-1 β and IL-8 and idiopathic infertility, but there was no correlation between bacterial vaginosis and ART outcomes. However, all patients in this study were treated for four days post trans-vaginal oocyte retrieval with antimicrobials, which may have affected ART outcomes.

The presence of infection may also modify cytokine expression, leading to altered reproductive function and adverse ART outcomes. To date, no study has investigated the levels of cytokines present in infected follicular fluid, even though altered immunological profiles within follicular fluid in response to microbial presence may be another cause of unexplained infertility.

Table 2.6 Cytokines associated with ART and pregnancy outcome

Cytokine	Tissue	Level of expression compared to normal	Outcomes	References
CD3+/CD4+[1] TNF α	Peripheral blood	↑	Implantation failure in ART	Ng *et al*., 2002
CD3+/CD8+[1] IL-10	Peripheral blood	↑	Implantation failure in ART	Ng *et al*., 2002
IFN γ, TNF α	Endometrium	↓ IFN γ and TNF α	Recurrent miscarriage	Shimada *et al*., 2003
IL-1	Endometrium	Significant ↑	Implantation success Placental development	Yin *et al.*, 2006
IL-10	Peripheral blood	↓	Idiopathic infertility	Ginsburg *et al*., 2005
IL-11 (epithelial cell)	Endometrium	↓ H score[2] for IHC[3]	Infertile Recurrent miscarriage	Linjawi *et al.*, 2004
IL-18	Plasma	↓	Recurrent miscarriage	Wilson *et al*., 2004 a
IL-18	Plasma	↑	Ist trimester miscarriage	Wilson *et al*., 2004 b
IL-1 Ra	Plasma	↑	Good quality embryos Increased clinical pregnancy	Spandorfer *et al*., 2003
IL-1 β	Plasma	↓	Good quality embryos Increased clinical pregnancy	Spandorfer *et al*., 2003
IL-6	Endometrium	↓	Recurrent miscarriage	Lim *et al.*, 2000
TGF β1	Plasma	↑	Recurrent miscarriage	Ogasawara *et al*., 2000
TNF β, IFN γ, IL-2, IL-12	Endometrium	↑ IFN γ and IL-12	Recurrent miscarriage	Lim *et al.*, 2000

[1]T helper cells and cytotoxic T cells (CD3+/CD4+/CD8+), [2]H score is a measure of immunoreactivity, [3] immunhistochemistry (IHC).

The literature highlights that the upper genital tract supports the growth of a diverse range of microorganisms that are able to: (1) replicate, secrete toxins and/or metabolites that can directly damage the genital tract mucosa and (2) induce an immune response, which can progress to excessive inflammation, tissue damage, and scarring. The formation of biofilms offers a microbial population protection and facilitates persistent growth, which may explain why many genital tract infections are asymptomatic. Characterisation of the microorganisms present within ovarian follicular fluid and the immune response to their presence will serve to enhance our understanding of the potential impact of UGT microbial colonisation on infertility. Knowledge of the microorganisms, their growth phenotype (planktonic or biofilm), the immune response they elicit and whether they are associated with colonisation, or infection (acute or chronic) of the genital tract may inform treatment for infertile couples.

Table 2.7 Alterations in follicular fluid cytokine concentrations and ART outcomes

Cytokine	Level of expression	Outcomes	References
EGF	↓↑ 0.2 – 10.3 (pg / mL)	Not indicative of ICSI outcome	Asimakopoulos *et al.*, 2006
FGF	↓↑ 20.1 – 391 (ng/mL)	Not indicative of ICSI outcome	Asimakopoulos *et al.*, 2008
GMCSF	↑ 57.7 ± 11.7 (pg / mL)	Not indicative of ICSI outcome	Salmassi, 2005
IGF1[1]	↓↑ 9.6 – 28 ng/mL	Not indicative of ICSI outcome	Asimakopoulos *et al.*, 2008
IGF2	↑ 83.7 ± 14.4 (ng/mL)	Good quality embryos	Wang *et al.*, 2006
IGFBP[2]-1	↑ 73.2 ± 41.7 (ng/mL)	Good quality embryos	Wang *et al.*, 2006
IGFBP-3	↑ 1784.9 ± 519.3 (ng/mL)	Successful fertilisation	Wang *et al.*, 2006
IGFBP-4	↑ 90.2 ± 39.0 (ng/mL)	Successful fertilisation	Wang *et al.*, 2006
IL-1	↓ 21.9 ± 9.2 (pg / mL)	Clinical pregnancy	Mendoza *et al.*, 2002
IL-1 α	↑ 7.9 – 19.4 (pg / mL)	Successful ART outcome Clinical pregnancy	Karagouni *et al.*, 1998
IL-1 β	↑ 32.5 – 111.7 (pg / mL)	Successful ART outcome Clinical pregnancy	Karagouni *et al.*, 1998
IL-6	↓↑ 1.7 – 12.2 (pg / mL)	No effect on ICSI outcome	Hammadeh *et al.*, 2003
IL-8	Present	Role in ovulation No correlation with outcome	Gazvani *et al.*, 2000
IL-12	Present	Poor fertilisation and implantation No pregnancy Poor ART outcome	Gazvani *et al.*, 2000
IL-12/23 common p40 subunit	↑ 58.3 – 133.6 (pg / mL)	Oocyte present Successful fertilisation Successful ET	Vujisic *et al.*, 2006
IL-15	↓ 0.01 – 1.7 (pg / mL)	Clinical pregnancy No correlation with oocyte presence	Vujisic *et al.*,2006
IL-15	↑ 0.9 – 2.2 (pg / mL)	Failure to achieve a pregnancy	Vujisic *et al.*, 2006
IL-18	↓25.1 – 50.3 (pg / mL)	Not correlated with oocyte presence No indication of ART outcome	Vujisic *et al.*, 2006
IL-18	↑ 37.9 – 55.5 (pg / mL)	Good quality embryos Successful ET	Vujisic *et al.*, 2006
Leptin	↑ 682.55 – 625.05 (ng/mL)	Failed conception	Asimakopoulos *et al.*, 2005
MIP-3αβ	↑ 5862 – 6700 (pg / mL)	Mature oocytes	Kawano *et al.*, 2004
PDGF	↑ 387.6 ± 357.0 (pg / mL)	Higher responder to ovulation induction	Hammadeh *et al.*, 2003
PDGF[3]	↓ 249.8 ± 150.1 (pg / mL)	Decreased fertilisation	Hammadeh *et al.*, 2003
SCF[4]	↑ 388.3 – 859.8 (pg / mL)	Not a predictor of ICSI or pregnancy outcome	Hammadeh *et al.*, 2004
sICAM[5]1	↑158.7 – 264.6 (ng/mL)	Not a predictor of ICSI or pregnancy outcome	Hammadeh *et al.*, 2004
VEGF	↓ 4.409 ± 2387 (pg / mL)	Successful ART outcome	Barroso *et al.*, 1999, Friedman *et al.*, 1997
VEGF	↑ 549 – 1800 pg / mL	Failed conception	Asimakopoulos *et al.*, 2006

[1]Insulin like growth factor (IGF), [2]Insulin like growth factor binding protein (IGFBP), [3]Platelet-derived growth factor (PDGF), [4]Sertoli cell factor (SCF), [5]Secretory intercellular adhesion molecule (sICAM)

CHAPTER THREE

GENERAL METHODS

3.1 Specimen collection

From September 2007 to November 2008, 263 consenting couples commencing fully stimulated IVF cycles at Wesley-Monash IVF were enrolled in this study. Ethical approval for this study was obtained from the review boards of UnitingCare Health, Human Research Ethics Committee (HREC) and Queensland University of Technology HREC. All patients gave permission for researchers to access medical records to obtain their reproductive history and IVF outcomes. The IVF clinicians collected all clinical specimens (follicular fluids and vaginal swabs), the IVF scientists aliquotted the follicular fluid into pre-prepared vials containing glycerol, or protease inhibitor, prepared by the candidate. The candidate performed all assays reported in this thesis.

Patients in this study were cohorted based on the medically diagnosed cause of infertility. Fertile women participating in IVF cycles because their male partners were infertile were used as the 'fertile' control group. The remaining women were grouped according to an aetiology of infertility due to: (1) endometriosis, which was laparoscopically diagnosed by identification of endometrial explants within the pelvic cavity; (2) polycystic ovary syndrome, diagnosed by blood testing for LH and FSH levels and an ultrasound scan; (3) a history of genital tract infection, defined by positive testing for *C. trachomatis*, pelvic inflammatory disease or STI often coupled with tubal factor infertility or bilateral salpingectomy, or (4) idiopathic infertility if the female and male partners had undergone screening for infertility and no abnormalities were detected. However, the classification of infertility may be imprecise. In up to 30% of couples, both the female and the male partners have some abnormality detected. For this study on follicular fluid, we have focused only on the

female factors of infertility. Therefore, to the best of our knowledge, all women classified as 'fertile' had no abnormal fertility results.

Couples were enrolled in this study in the order in which they began IVF treatment and consented to be in this project. No efforts were made to control the distribution of group sizes based on the aetiology of infertility.

Trans-vaginal oocyte retrieval

From each female patient, the IVF clinician collected two vaginal swabs in addition to the follicular fluid collected at the time of oocyte retrieval. One vaginal swab was collected prior to cleansing of the vagina, which was tested for cytokines and a second (after cleansing of the vagina with sterile saline) was reserved for microbiological culture in experiments for this thesis. The IVF unit used a 'boost' protocol for controlled ovarian hyper-stimulation. A gonadotropin-releasing hormone agonist was administered from day 2 of the menstrual cycle and follicle-stimulating hormone was administered from day 4. Follicle size was monitored by trans-vaginal ultrasound scans and once multiple follicles reached 17 – 19 mm in diameter, the patient received an ovulatory dose of human chorionic gonadotropin. Oocytes were collected 36 hours later by trans-vaginal oocyte retrieval. Follicular fluid specimens and oocytes were collected at the time of standard trans-vaginal oocyte retrieval. Briefly, the ultrasound probe was wiped with sterile distilled water and finally a 70% ethanol isowipe. The ultrasound probe was covered with a disposable sheath. Trans-vaginal oocyte retrieval was performed using a sterile needle (K-OPS-1032-WMC Cook Medical Single Lumen Aspiration Needle, Brisbane, QLD) attached to a needle holder on a vaginal ultrasound probe. The vaginal wall was prepared with sterile saline to remove excess mucus and cellular debris. Immediately prior to oocyte

retrieval, the needle was flushed with follicle flush buffer (K-SIFB-100 Cook Medical – Follicle Flush Buffer (Cook Medical). For each study participant, the follicular fluid from the largest most accessible follicle in either the left or right ovary was aspirated first. Follicular fluid was aspirated directly into sterile test tubes in the operating theatre. This follicular fluid specimen was aseptically transferred to a sterile culture dish to determine if there was an oocyte present, the oocyte was transferred to a sterile culture dish and the follicular fluid was aliquotted: 1 mL was added to 1 mL 80% glycerol (to give a final concentration of 40%); 1 mL was added to 20 µL protease inhibitor cocktail (Sigma Aldrich, Castle Hill, NSW); and the remaining follicular fluid was transferred into a sterile 15 mL Falcon tube. Only follicular fluids containing an oocyte were tested for this study. Aliquots of semen and washed semen samples not used for insemination were also aliquotted into 80% glycerol. All specimens were batched and stored at -80° C at the Wesley Research Institute tissue bank.

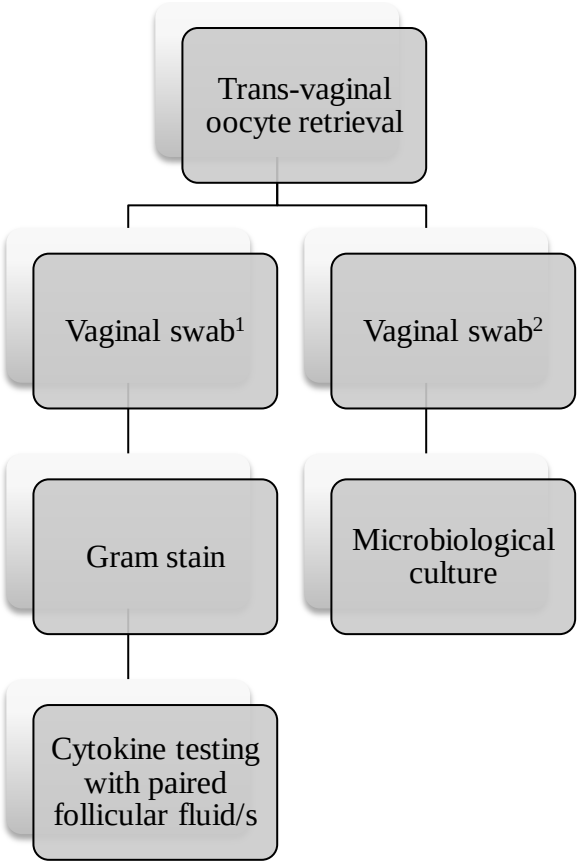


Figure 3.1 Flow chart for vaginal specimen collection during trans-vaginal oocyte retrieval for IVF
[1] Collected prior to normal saline vaginal cleanse, [2] collected after normal saline treatment of vaginal wall directly prior to oocyte retrieval.

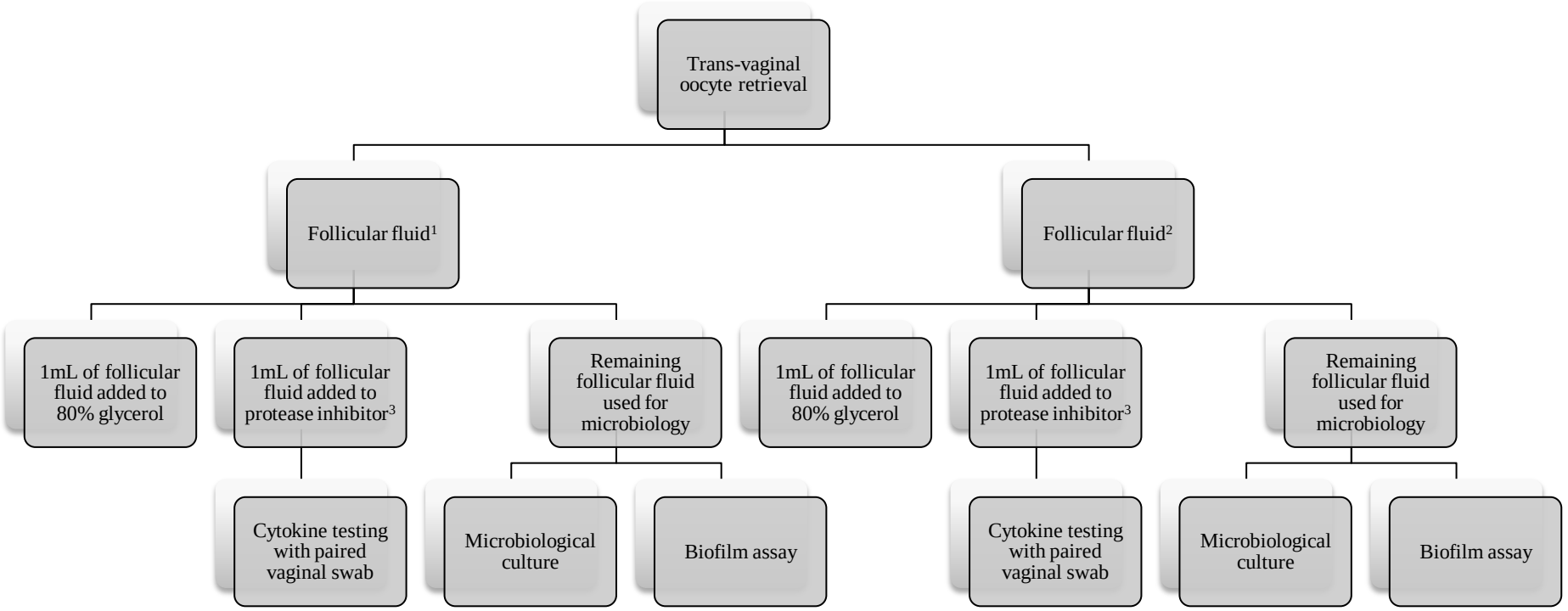


Figure 3.2 Flow chart for follicular fluid specimen collection and processing at the time of trans-vaginal oocyte retrieval for IVF
[1] Collected from the largest most accessible follicle in the left ovary, [2] Collected from the largest most accessible follicle in the right ovary, [3] Non-blood stained follicular fluids only

Semen preparation (performed by IVF scientists)

Density gradient medium was used to filter out cells and debris with centrifugation. On the day prior to oocyte retrieval, two flasks of PureSperm (Cook, Brisbane, QLD) culture media were prepared. A 27 mL volume of the 90% PureSperm (Cook) was added to 3 mL culture medium in a Falcon flask. A second flask was prepared by adding 13.5 mL of 45% PureSperm to 16.5 mL of culture medium. A 1 mL volume of 90% stock solution was dispensed into a conical based Falcon tube. A 1.5 mL layer of the 45% PureSperm mixture was gently layered on top of the base solution. Tubes of 90/45% PureSperm were dispensed and incubated overnight at 37° C in 6% CO_2. Following semen specimen production on the day of oocyte retrieval, semen was separated into two aliquots and each was layered over the top of the equilibrated PureSperm. The Falcon tube containing the semen and PureSperm was centrifuged at 200 × g for 15 minutes. The pellet was removed from the base of each tube with a sterile Pasteur pipette and transferred to a sterile 10 mL test tube and resuspended in 10 mL of culture medium. This mixture was centrifuged at 300 × g for 10 minutes. Following removal of the supernatant, the pellet was resuspended in 1 mL of culture medium. An abbreviated semen analysis was performed and the volume, concentration and percentage of motile spermatozoa were recorded. The sample was held in a heating block at 37° C or in an incubator under 6% CO_2 if not required immediately.

Insemination

Standard insemination

IVF scientists transferred one or two oocytes into a fertilisation well containing fertilisation medium (Cook) using a sterile glass pipette. Oocytes were inseminated

with 80000 motile spermatozoa per well using a pipette and sterile filter tip. Fertilisation dishes then were transferred to MINC incubators at 37° C under 6% CO_2.

Intracytoplasmic sperm injection (ICSI)

IVF scientists transferred a maximum of four oocytes to a single well in a 4-well dish (Nunc, Scoresby, VIC) containing 50 µL of hyalase and 950 µL of gamete wash buffer (Cook Medical). Cumulus masses were loosened by gentle pipetting up and down using a polished sterile glass pipette in the hyalase solution (Cook) for a maximum of 15 seconds. Oocytes then were transferred to a second well containing 1 mL of gamete wash buffer using a polished glass pipette with a smaller bore. The process was repeated for the remaining two wells until all cumulus cells were removed and the corona loosened. Oocytes were assessed on an inverted microscope at a total magnification of × 200 or × 300. Metaphase II (MII) oocytes were incubated for a further one hour prior to injection in fertilisation or gamete wash buffer. ICSI insemination was performed using a microinjector with holding and injection pipettes using spermatozoa diluted to a final concentration of one million per mL within fertilisation media.

Fertilisation and embryo cleavage

The fertilisation checks were performed between 16 and 19 hours post-insemination. The presence of two pronuclei and two polar bodies indicated normal fertilisation. The denuded oocytes were visualised for the presence of pronuclei using partial dark field microscopy on a stereo microscope. All fertilised oocytes were transferred to fresh culture medium for further culture. Day two and day three cleavage stage

embryos were assessed and graded using an inverted microscope with Hoffman or Nomarski optics. Cleavage stage embryos were graded by the cell stage and degree of fragmentation. Blastocyst grading was based on the degree of expansion and hatching, inner cell mass and trophectoderm gradings (Wesley Monash IVF).

Embryo transfer

Embryo transfer was performed by the IVF clinician on day three, four or five after *in vitro* culture. After assessment, the embryo (s) was selected for transfer. The selected embryo (s) was placed into an embryo transfer well containing the stage appropriate culture medium (cleavage or blastocyst). The embryo transfer was performed by the IVF clinician using a 1 mL syringe containing patient media, attached to an IVF transfer catheter (K-Jet 7019-SIVF, Cook). All women were administered progesterone luteal support commencing on the day of trans-vaginal oocyte retrieval and continuing until at least the day of the serological pregnancy test.

3.2 Microbial culture and colony identification

Follicular fluids and vaginal specimens were thawed on ice prior to culture. Using a sterile 1 µL or 10 µL calibrated inoculating loop (Becton Dickinson, North Ryde, NSW) a range of culture media (horse blood agar, chocolate I agar, MacConkey agar, Sabouraud dextrose agar, de Man Rogosa Sharpe agar, anaerobic blood agar) (Oxoid, Adelaide, SA) was inoculated with undiluted follicular fluid (onto the initial inoculum area of each agar plate) using the 16-streak technique. Specimens were also inoculated into thioglycollate broth (Oxoid). Plate media were incubated either aerobically, in 5% CO_2 or anaerobically in anaerobic jars (Oxoid anaerogen, 2.5 L,

Oxoid) at 37° C. Colony forming units (CFUs) on plates incubated aerobically were semi-quantified after 24 hours, whilst those on plates incubated under CO_2 or anaerobically were counted after 24 hours and again at 48 hours (Isenberg, 2009). The anaerobic plates and thioglycollate broths were examined and CFUs enumerated at 48 hours and then every second day up to 14 days. Semi-quantification of the colonies was performed by counting the approximate numbers of colonies on the plate (Table 3.1) and multiplying that number by the dilution factor appropriate for the inoculating loop used (10^3 for a 1 µL loop or 10^2 for a 10 µL loop) to give the CFU / mL. Each different colony type from all plates was Gram-stained and subcultured for biochemical identification. Where quantification was not required, a sterile swab (Interpath, Heidelberg West, Victoria) was used to inoculate a primary inocula zone on an agar plate, which was then diluted using the 16-streak method.

Table 3.1 Enumeration of CFU / mL of follicular fluid or vaginal secretion by semi-quantification

Growth on each set of streaks	Number of colonies counted	CFU / mL
Primary inoculum	1	10^3
1+	1-10	10^4
2+	10-100	10^5
3+	100-1000	10^6
4+	>1000	$>10^6$

Gram-negative rods were identified using API20E identification strips (Biomerieux, Baulkham Hills, NSW), and catalase positive Gram-positive cocci were identified by Staphylase (Oxoid) and rabbit plasma tube coagulase testing (Becton Dickinson).

Identification of pigmented catalase positive, Gram-positive cocci suspected to be of the Micrococcaceae family were identified by 16S ribosomal RNA (rRNA) polymerase chain reaction (PCR) assay followed by sequencing of the amplicon at the Australian Genome Research Facility (AGRF, University of Queensland, Brisbane, Qld). Catalase negative Gram-positive cocci from the Streptococcus family were identified by Lancefield grouping (Oxoid) and bacitracin (Oxoid) or optochin (Oxoid) where appropriate. Anaerobic isolates were identified by API20A (Biomerieux) identification strips followed by a 16S rRNA PCR assay and sequencing of amplicons (AGRF, Queensland) if isolates scored a poor API T-value or percentage identification.

Colony morphology, simple staining with lactophenol cotton blue (Pathtech, Preston VIC) and yeast microrings, Microring YT (Medical Wire and Equipment Company, Victory Gardens, New Jersey) were used to identify *Candida* spp.

3.3 DNA extraction

Prior to the 16S rRNA PCR assay each colony was first resuspended in 100 µL of 100 µg/mL proteinase K (Sigma Aldrich, Castle Hill, NSW) and 100 µL of sterile distilled water. The suspension was vortexed and then incubated at 37° C for one hour. DNA was extracted using a Qiagen QiAMP Mini DNA extraction kit (Qiagen, Doncaster, VIC) as per the manufacturer's instruction for extraction from blood and body fluids. The final DNA elution was into 50 µL of sterile distilled water.

3.4 16S rRNA PCR amplification of follicular fluid

The 16S rRNA PCR assay was performed using the previously published primers, RW01 (5′-AAC TGG AGG AAG GTG GGG AT-3′) and DG74 (5′-AGG AGG TGA TCC AAC CGC A-3′) and PCR cycling conditions (Greisen *et al.*, 1994), with a degeneracy (T/C) incorporated into the 3' end of the upstream RW01 primer. The PCR master mix included: 1 X buffer; 200 μM of dNTPs; 0.5 μM of each primer; 5 U *Taq* polymerase (Roche, Castle Hill, NSW) and 8 μL of the extracted bacterial DNA to a final volume of 50 μL. PCR cycling conditions included: an initial denaturation at 95° C for 15 minutes; followed by 25 cycles of denaturation at 94° C for 20 seconds, primer annealing at 55° C for 20 seconds, extension at 72° C for 20 seconds; and a final extension step at 72° C for 10 minutes (PTC-200, Peltier Thermal Cycler, BioRad, Gladesville, NSW).

3.5 Agarose electrophoresis

PCR amplicons were run on 2% agarose gels prepared in tris borate EDTA (TBE) buffer. Agarose was dissolved in the TBE buffer with ethidium bromide added to a final concentration of 1μg/mL (Sigma-Aldrich). Amplicons were electrophoresed at 100 volts for 60 minutes and visualised under ultraviolet light.

3.6 Sequencing

All PCR products were sequenced by the Australian Genome Research Facility (AGRF, St. Lucia, QLD). The PCR products were first purified using the Roche HighPure kit as per the manufacturer's instructions. The final elution was into 50 μL of sterile distilled water. A purified DNA service was used as per AGRF guidelines. Briefly, two reactions (forward and reverse) were prepared using high quality

(OD_{260}/OD_{280} 1.8-2.0) genomic DNA (6-18 ng) and 6.4 pmol of a single PCR primer in a final volume of 12 µL. AGRF performed the PCR sequencing reaction: an initial denaturation at 96° C for 2 minutes for one cycle, followed by 30 cycles of denaturation at 96° C for 10 seconds, primer annealing at 50° C for 5 seconds, extension at 60° C for 4 minutes, and then held at 4° C. AGRF used a DNA Big Dye 3 sequencing technology (BDT) labelling sequencing platform. The sequence, obtained in a FASTA format then was entered into the Basic Local Alignment Search Tool (BLAST, NCBI) for identification of clinical isolates.

CHAPTER FOUR

MICROBIOLOGICAL CHARACTERISATION OF HUMAN FOLLICULAR FLUID

4.1 Introduction

The presence of microorganisms in female reproductive tissues has been associated with adverse pregnancy outcomes after both natural as well as IVF conceptions. Miscarriage, stillbirth, preterm rupture of membranes and preterm delivery are some of the adverse outcomes reported and have been connected with recovery of microorganisms from amniotic fluid, placental membranes or products of conception (McClure and Goldenberg, 2009). The presence of microorganisms within the endometrial cavity can cause the activation of both maternal and foetal immune responses. The resultant inflammatory environment can lead to foetal demise *in utero* by direct infection of the foetus, or can initiate early labour by cytokine activation of prostaglandins, leading to cervical ripening and uterine contractions (Romero *et al.*, 2001).

4.2 Background

Numerous studies have been undertaken to determine if the female upper genital tract is normally sterile, in the absence of a symptomatic infection and increasingly, reports indicate that microorganisms are present within the female upper genital tract of health asymptomatic women (Saltes *et al.*, 1995, Cicinelli *et al.*, 2009, Spence *et al.*, 1982, Andrews *et al.*, 2005). However, investigations of microorganisms within follicular fluid have been limited. One previous study has reported the presence of bacteria within follicular fluid collected at the time of trans-vaginal oocyte retrieval, but these bacteria were not associated with adverse IVF outcomes (Cottell *et al.*, 1996). In a preliminary study in our laboratory, a fibrin clot was observed in a follicular fluid. Subsequently, the follicular fluids of 33 IVF patients were cultured and bacteria were isolated from 22 (66%) of these follicular fluid samples. It was

proposed that the presence of bacteria in follicular fluid was associated with adverse IVF outcomes (Knox, Personal communication). It was then hypothesised that microorganisms present within the upper genital tract may be a potential source of invasive infection, whereby the microbes themselves, or the immunological response to their presence, may lead to adverse pregnancy outcomes. In order to test this hypothesis, follicular fluid collected from IVF patients at the time of trans-vaginal oocyte retrieval was analysed to identify microorganisms present. Microorganisms can gain access to the female upper genital tract by ascension from the lower genital tract, by haematogenous dissemination, in seminal fluid or by iatrogenic inoculation. Paired vaginal specimens were tested in parallel to determine whether microorganisms were introduced into follicular fluid at the time of trans-vaginal oocyte retrieval, or had independently colonised the follicular fluid prior to oocyte retrieval. The presence of bacteria within follicular fluid then was correlated with IVF outcomes.

4.3 Materials and Methods

4.3.1 Experimental design

The results presented in this thesis were based on microbiological studies of a cohort of 263 women. In this chapter, we have assessed and described the microflora of the entire cohort (n = 263). One woman had no microorganisms detected in her follicular fluid by either traditional culture or molecular microbiology techniques. This woman was excluded from further analysis because our hypothesis may not be tested in the absence of microorganisms. For the remaining 262 women, the microorganisms identified in follicular fluid were analysed to determine if there was a relationship between the microbial flora and IVF outcomes.

4.3.2 Specimen collection

Specimen collection and storage was performed as previously described in the General Methods chapter (Section 3.1). Two types of specimens were collected from each woman, follicular fluid samples (n = 463), and vaginal swab specimens (n = 263) were cultured for the detection and identification of microbial species present.

4.3.3 Culture and colony identification

Culture and identification of clinical isolates was performed as previously described in the General Methods chapter of this thesis (Section 3.2).

4.3.4 16S rRNA PCR assay testing of follicular fluid

PCR amplification was performed as described in Chapter Three (Sections 3.3 – 3.6).

4.3.5 Detection of *Mycoplasma* spp. and *Ureaplasma* spp. by real-time PCR of follicular fluid

Real-time PCR assays were performed on DNA extracted from follicular fluid to detect *Mycoplasma genitalium*, *M. hominis*, *U. parvum*, and *U. urealyticum*, using previously published primer pairs (Chalker *et al.*, 2009, Pascual *et al.*, 2010, Xiao *et al.*, 2010). The PCR primers used are described in Table 4.1. These species have been previously isolated within the genital tract, but are difficult to recover by routine bacteriological culture, the ureaplasmas requiring specialised media, whilst *M. genitalium* is particularly fastidious. High-resolution melt (HRM) curve analysis was subsequently performed to discriminate each of these species based on the shape of the HRM curve (Vossen *et al.*, 2009). Melt curve normalisation and comparison software was used to distinguish between bacterial species with similar melt curves (Qiagen).

Table 4.1 Real-time PCR primers for the detection of the genital mycoplasmas

Organism	Gene	Forward primer	Reverse primer	Amplicon	Reference
		5’ 3’	5’ 3’		
M. genitalium	mg219 NC000908	GAGAAATACCTTGATGGTCAGCAA	GTTAATATCATATAAAGCTCTACCGTTGTTATC	251 bp	Chalker *et al*., 2009
M. hominis	16s rRNA AF443616	CAATGGCTAATGCCGGATACG	GGTACCGTCAGTCTGCAATC	101 bp	Pascual *et al*., 2010
U. parvum	UP063 NP077893	TGCGGTGTTTGTGAACT	TGATCAAACTGATATCGCAATTATAGA	152 bp	Xiao *et al*., 2010
U. urealyticum	UUR10_0680 NZAAYN00000000	GGATTTGTTAGATATCGTCAAGG	TCATCTTTTAAAGCTCCACATTATTAGT	152 bp	Xiao *et al*., 2010

The real-time PCR mastermix for each primer comprised: 12.5 μL of 2× Sybr green Platinum PCR master mix (Invitrogen, Mulgrave, VIC), 0.5 μM of each primer and 1 μL of DNA template and sterile H_2O to a final reaction volume of 25 μL. Identical real-time PCR cycling conditions were used for each primer pair. Cycling conditions included: an initial hold at 50° C for 5 minutes followed by denaturation at 95° C for 10 minutes and then; followed by 55 cycles of denaturation at 95° C for 10 seconds, primer annealing at 52° C for 20 seconds; and a final extension step at 72° C for 20 seconds. HRM analyses of all samples was performed post-run by ramping from 60° C to 90° C, with fluorescence data acquisition in 0.05° C increments. HRM analyses were carried out using Rotor-Gene 6000 1.7.34 software (Qiagen). Both conventional and normalised dissociation plots were generated. The normalised dissociation plot was used to produce difference graphs in order to quantitatively determine the sample deviation relative to the control samples. The nucleotide composition of the PCR product and the initial template concentration determined the shape of the curve and the melting temperature of single and double stranded DNA. Based on the melting temperature and shape of the curve as compared to controls, the genotype of the unknown specimen can be determined. HRM analysis was undertaken post-run on the amplified DNA products. Species identification (genotype) was determined as 'same' by a cut-off of ≤ 5 U in the HRM difference graph relative to a specified, normalised genus/species (Price *et al*., 2007) (Figure 4.2). Genotypes were classified as 'different' if the fluorescence reading criterion was > 5 U. Visual inspection of normalised and conventional dissociation plots was also used to confirm the genotypes (Figure 4.2). Four positive controls, clinical isolates of *M. genitalium*, *M. hominis* (from D. Wiley, Sir Albert Sakzewski Virus Research Centre, Brisbane) and reference serovars of *U. parvum* (serovar 3), and *U. urealyticum* (serovar 8)

(courtesy of H. Watson, University of Alabama, Alabama) were included in each run to provide internal controls for comparative analysis. All samples were tested in duplicate to ensure reproducibility of the melt curves and to determine appropriate cut-offs for each genotype. A no-template master mix tube was also included as a negative control in each run.

4.3.6 Statistical analysis

Statistical analyses were performed using SPSS version 17 for Windows XP. Multinomial logistic regression was used to model the relationship between categorical outcomes (fertilisation, embryo discard, embryo transfer, and pregnancy rates) and explanatory variables (the presence of colonising or contaminating microbial species within follicular fluid). In the regression analyses, a p-value of $p < 0.05$ was considered statistically significant.

4.4 Results

4.4.1 Patient demographics

The mean age of women in this study was 37 years ± 4 years. There were no statistical differences in the mean age of fertile women (37 ± 4 years) and infertile women (37 ± 6 years, $p > 0.05$). There was no difference in the number of prior ART treatment cycles undertaken for fertile and infertile women. Fertile women participated in an average of one (range 0-4 ± 1 cycle) previous trans-vaginal oocyte retrieval procedure compared to infertile women who had also participated in one prior cycle (range 0 - 5 ± 1 cycle, $p > 0.05$). Furthermore, there was no difference in the number of oocytes collected at the time of retrieval for fertile (11 ± 6) or infertile women (11 ± 6, $p > 0.05$)

4.4.2 Culture and colony identification of species isolated from follicular fluid and vaginal secretions

The culture analyses revealed that cultivable bacterial species (1 – 5 species) were present in 99% of follicular fluids tested. For only one woman no microorganisms were detected within the single follicular fluid sample that was available for testing. The results for this woman/couple were excluded from all statistical analyses. Microorganisms also were detected within 100% of cultured vaginal secretions. A range of microorganisms was isolated from follicular fluid from the left ovary, and/or right ovary and from the vagina of women enrolled in this study. For women in this study, their follicular fluids were classified as: (1) 'colonised' (75/262, 29%) if microorganisms were detected within the follicular fluid, but not in the vagina (at the time of oocyte retrieval); or (2) 'contaminated' (187/262, 71%), if microorganisms detected in the vagina at the time of trans-vaginal oocyte retrieval were also detected within the follicular fluid. We note that the vagina may not be the only source of the contaminating microorganisms detected in the follicular fluids tested in this study; however, assessment of the source of follicular fluid microorganisms was restricted by the sampling methods routinely employed for oocyte retrieval at Wesley Monash IVF, namely through trans-vaginal aspiration. To account for the sampling methods available in this study, we tested both the follicular fluid and paired vaginal specimens in parallel to allow the use of the definition that was adopted in this study. Consequently we have reported 'colonised' follicular fluid only when the same microbial species were not also detected in the vaginal secretions. The basis of our definition is that colonisation is a natural process within body cavities, whilst contamination represents a possibility for improvement in IVF techniques. Both fertile women (n = 60) (couples with infertile male partners) and the group of infertile women (n = 202) with various causes of infertility, demonstrated both

colonisation and contamination of follicular fluid specimens (Table 4.2). The percentage of 'fertile' women with colonized follicular fluid was 27%. The rates of colonisation in infertile women ranged from 24 – 37% (Table 4.2) and there was no difference in the follicular fluid colonisation rate for infertile women (any cause of infertility) ($p > 0.05$). Culture analysis revealed that 0 - 5 different microbial species were detected within the left follicular fluid specimens and from 0 - 5 species were detected within the right follicular fluid specimens (Table 4.3). More microorganisms were isolated from the left follicular fluid (mean = 2.6 ± 1.3) when compared to the right follicular fluid (mean = 2.0 ± 1.4) ($p < 0.0001$) (Table 4.3). Culture of vaginal swabs detected 1 - 11 species within the lower genital tract (Figure 4.1 A). There were subtle differences in the most prevalent microbial species detected in the left and right follicular fluids and in the vaginal secretions in both fertile and infertile women (Figure 4.1 B - F). *Lactobacillus* spp. and *Bifodobacterium* spp. were the most prevalent species detected in the genital tract specimens of all women (Figures 4.1 B - F). For all women, *Actinomyces* spp. and *Staphylococcus* spp. were also among the most prevalent species isolated except in e women diagnosed with idiopathic infertility (Figure 4.1 B - F).

Table 4.2 Number of women with colonised or contaminated follicular fluid and their corresponding cause of infertility

Aetiology	Endometriosis (n = 49)	Polycystic ovary syndrome (n = 48)	Genital infection (n = 39)	Male factor[1] (n = 60)	Idiopathic (n = 66)	Total (n = 262)
Colonised follicular fluid	18 (37%)	14 (29%)	11 (28%)	16 (27%)	16 (24%)	75 (29%)
Contaminated follicular fluid	31 (63%)	34 (71%)	28 (72%)	44 (73%)	50 (76%)	187 (71%)

[1] 'Fertile' women with infertile male partners

Table 4.3 The overall number of microbial species detected within the follicular fluid from the left and/or right follicle(s) and from the vagina of the 262 women with culture-positive follicular fluid

Number of microbial species detected by culture	Left follicle (n = 248)	Right follicle (n = 217)	p-value	Vagina (n = 262)
0	24/248 (10%)	41/217 (19%)	0.004	0/262 (0%)
1	31/248 (13%)	21/217 (10%)	> 0.05	2/262 (1%)
2	52/248 (21%)	76/217 (34%)	0.0007	36/262 (14%)
3	72/248 (29%)	60/217 (28%)	> 0.05	10/262 (4%)
4	58/248 (23%)	15/217 (7%)	< 0.0001	23/262 (9%)
5	11/248 (4%)	4/217 (2%)	> 0.05	35/262 (13%)
6	0 (0%)	0 (0%)	n/a	40/262 (15%)
7	0 (0%)	0 (0%)	n/a	24/262 (9%)
8	0 (0%)	0 (0%)	n/a	40/262 (15%)
9	0 (0%)	0 (0%)	n/a	18/262 (7%)
10	0 (0%)	0 (0%)	n/a	17/262 (6%)
11	0 (0%)	0 (0%)	n/a	17/262 (6%)
Mean ±SD	2.6 ±1.3	2.0 ±1.4	< 0.0001	

A. Overall microbial prevalence for follicular fluid and vagina

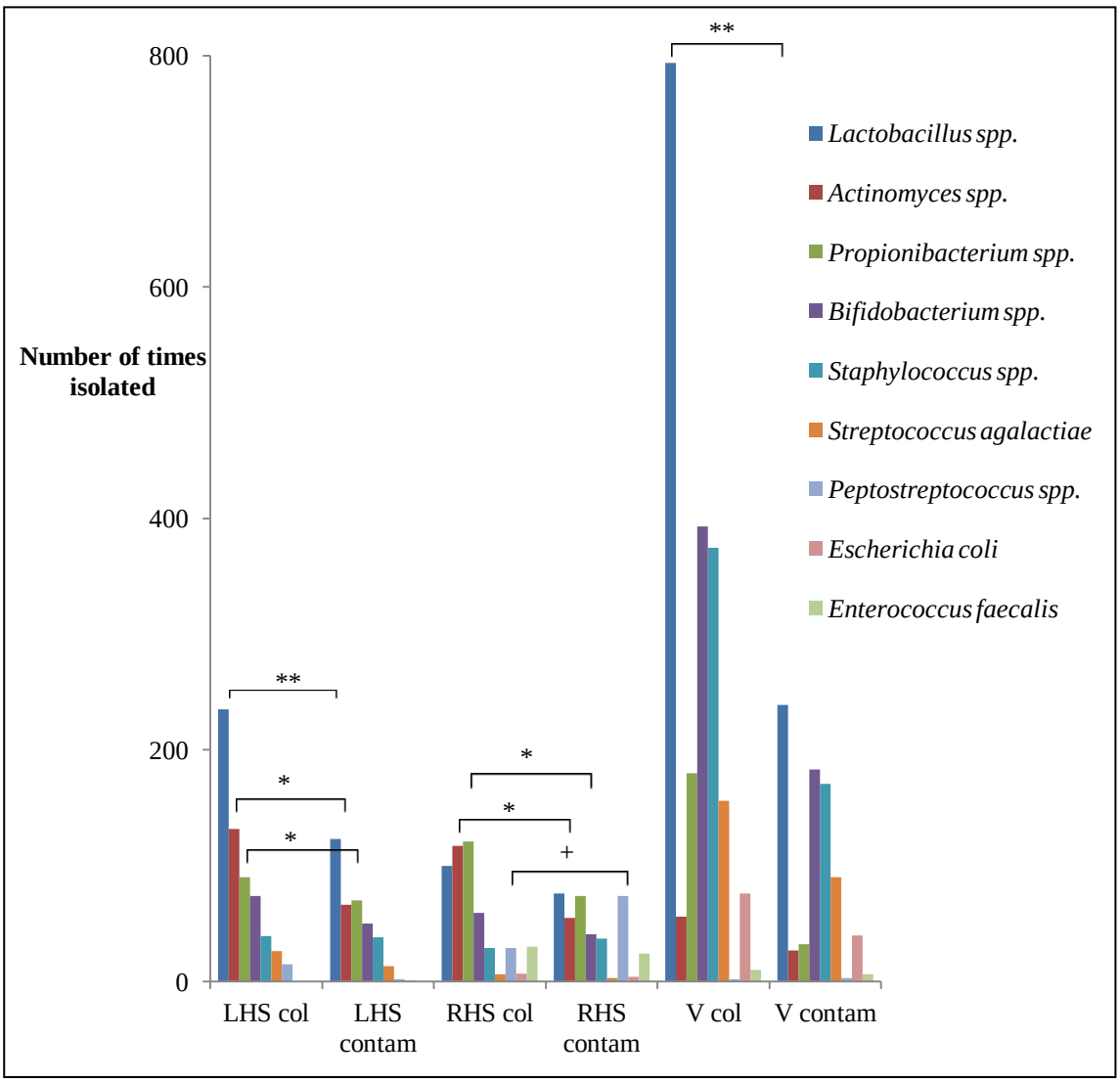


Figure 4.1 The most prevalent microbial species detected from the left or right follicle and vagina in women with colonised and contaminated follicular fluid. Figure 4.1 A. The most prevalent bacterial species identified from follicular fluid collected from the left and right ovary and the vagina of women with either colonised or contaminated follicular fluid. Each of the three anatomical sites was grouped according to whether the follicular fluid tested was defined as colonised or contaminated. The vaginal secretions from women with colonised follicular fluid had a higher number of bacterial isolates when compared to women with contaminated follicular fluid specimens; however, the same species were detected in both groups of women.

LHS=Left follicle, col = colonised, contam = contaminated, RHS = right follicle, V = vagina
* = $p < 0.05$ coloniser, ** = $p < 0.01$ coloniser, + = $p < 0.05$ contaminant

B. Overall microbial prevalence for fertile women

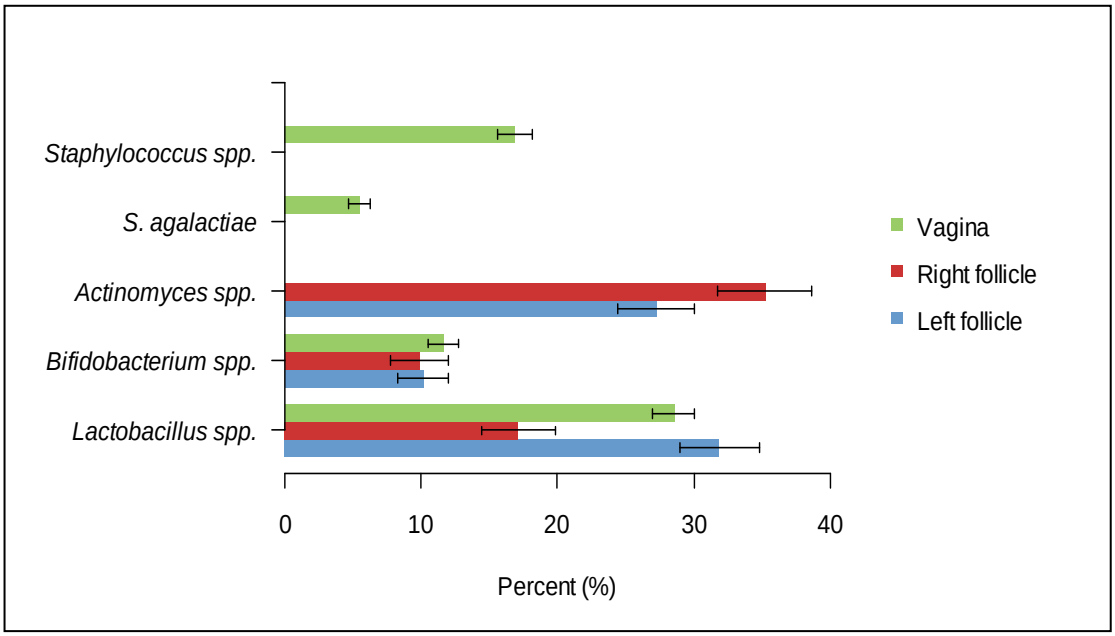
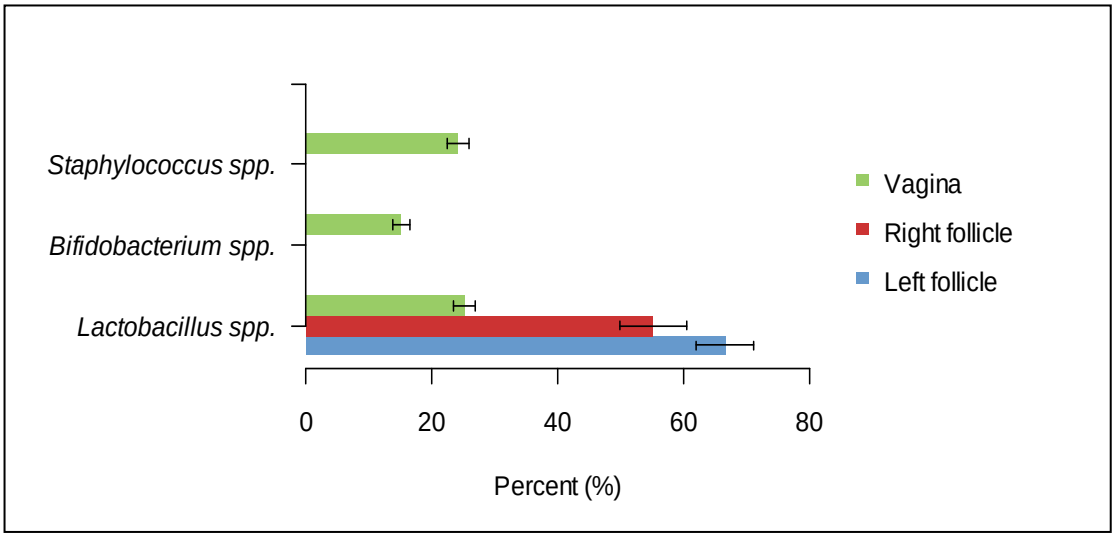
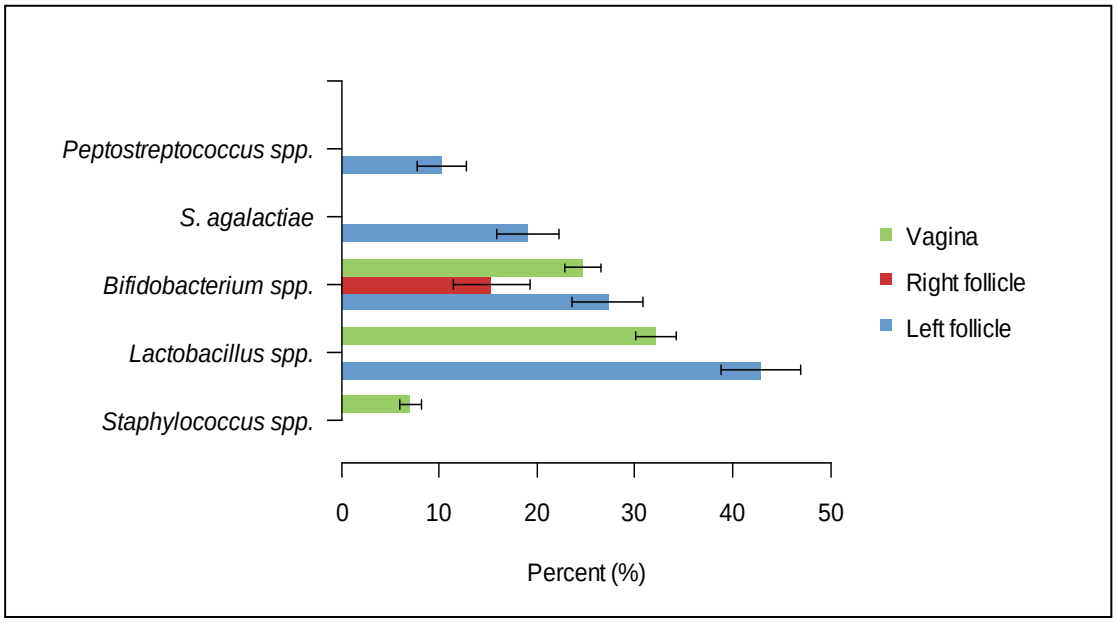


Figure 4.2 B-F The most prevalent species detected from the left and right follicular fluids and vagina from women in this study are stratified based on the diagnosed cause of infertility. Bars represent one standard error.

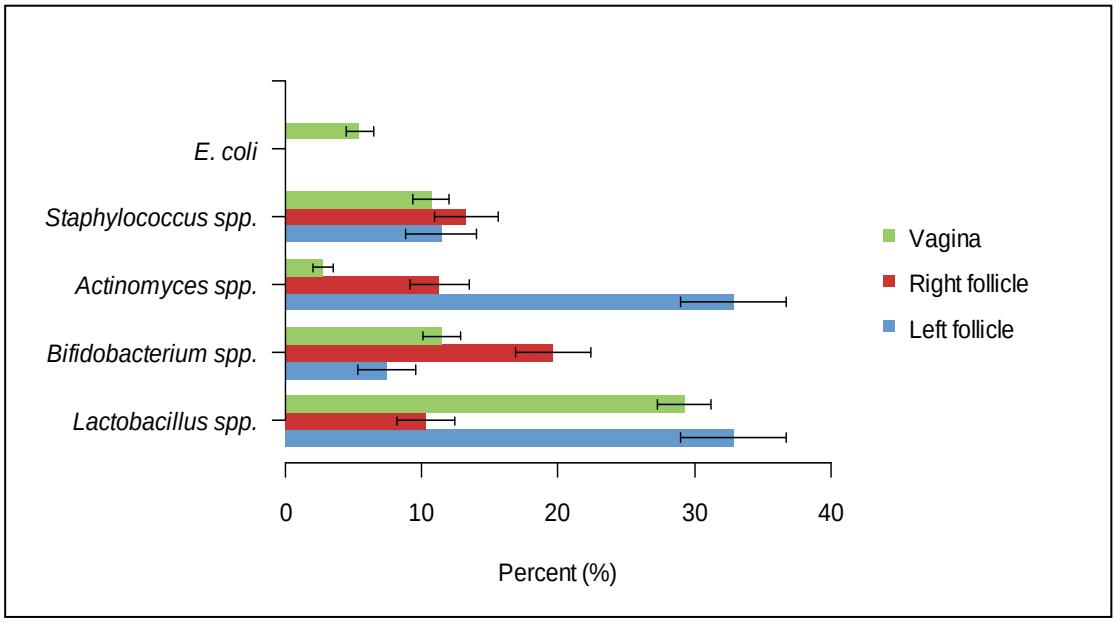
C. Overall microbial prevalence for women with endometriosis



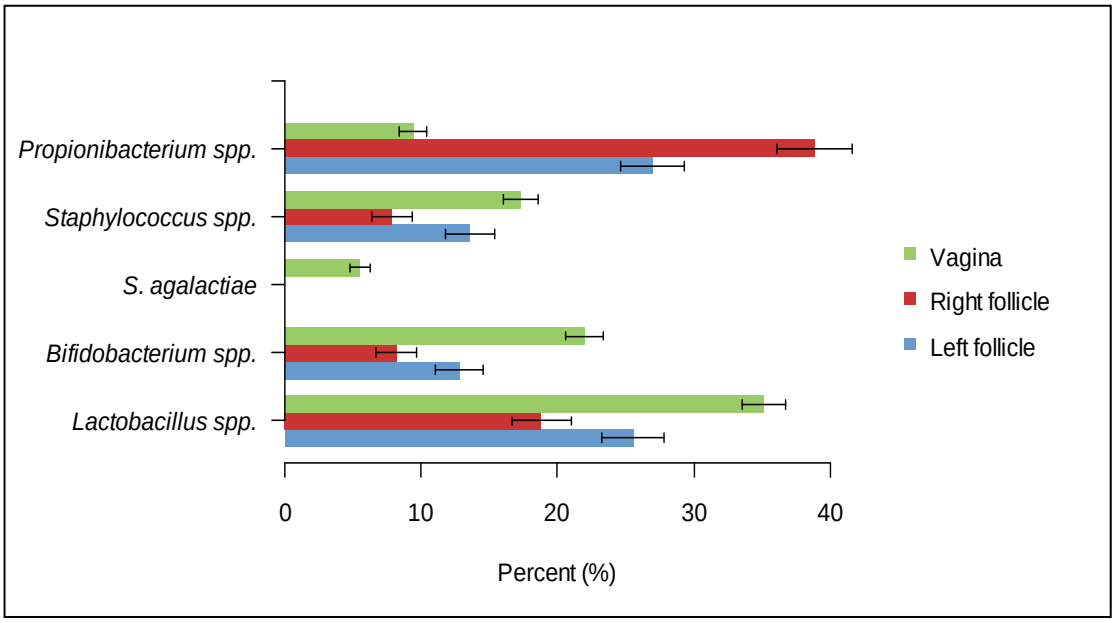
D. Overall microbial prevalence for women with polycystic ovary syndrome



E. Overall microbial prevalence for women with a history of genital tract infection



F. Overall microbial prevalence for women with idiopathic infertility



4.4.3 Bacterial load

The effect of microbial load was also considered in this study. Due to the polymicrobial nature of microorganisms detected within the follicular fluid and vaginal secretions, exact quantification of each bacterial species was not possible. A semi-quantitative method was therefore adopted to assess the overall prevalence and diversity of microorganisms. Table 4.4 highlights that most species were isolated at concentrations ranging from 10^3 CFU / mL to $>10^6$ CFU / mL in both colonised and contaminated follicular fluids. There appeared to be no correlations between the microbial loads in follicular fluid either in colonised or in contaminated follicular fluid(s) and (1) the aetiology of infertility or (2) the IVF outcomes.

In left follicular fluids, *Lactobacillus* spp. was isolated significantly more times from colonised compared to contaminated follicular fluids ($p = 0.004$) (Figure 4.1 A). This was also true for *Actinomyces* spp. ($p = 0.01$) (Figure 4.1 A). In the follicular fluids

collected from the right ovary, *Actinomyces* spp. and *Propionibacterium* spp. were the only species isolated more frequently from the colonised fluid than from contaminated follicular fluids ($p = 0.0001$ and 0.01 respectively). *Peptostreptococccus* spp. was isolated more frequently from contaminated follicular fluid from the right ovary than from colonised follicular fluid from the right ovary ($p < 0.001$).

The women in this study had 1 - 11 different cultivable microbial species comprising the vaginal microflora (Table 4.3). In addition, the *Lactobacillus* spp., *Bifidobacterium* spp., *Staphylococcus* spp., and *S. agalactiae* were the most prevalent species identified as vaginal flora in women with colonised and contaminated follicular fluid (Figure 4.1 A).

4.4.4 Colonised follicular fluid, asymmetrical colonisation and IVF outcomes

Logistic regression analysis was used to test for the degree of independence between colonisation of follicular fluid (either left or right) and aetiology of infertility. Endometriosis, PCOS, genital tract infection, male factor and idiopathic aetiologies were considered. Colonisation of left follicular fluid and endometriosis were found to be dependent ($p < 0.05$). There were no other statistically significant results (data not shown).

Decreased embryo transfer rates were observed in women with colonised follicular fluid (fertile women, $p = 0.002$ and women with endometriosis, $p = 0.02$) (Table 4.5). Table 4.5 also shows pregnancy rates in women with colonised follicular fluid and pregnancy rates (fertile women, $p = 0.007$, and women with infertility due to

endometriosis, $p = 0.014$ and polycystic ovary syndrome, $p = 0.032$). The aetiology of infertility was not associated with decreased pregnancy rates for infertile women with a history of genital tract infection or idiopathic infertility ($p > 0.05$) (Table 4.5). Embryo discard rates were higher for fertile women with contaminated follicular fluid ($p = 0.005$) and for women with endometriosis with colonised follicular fluid ($p < 0.0001$) (Table 4.5). The regression model also found that overall, follicular fluid colonisation does not differentiate between fertilisation rates for infertile women with a diagnosis of endometriosis, polycystic ovary syndrome or a history of upper genital tract infection when normalised against the control group of 'fertile' women (with infertile partners) (Table 4.5). Overall, colonised follicular fluid was associated with a decrease in the embryo transfer and pregnancy rates for all women (fertile and infertile) ($p < 0.05$) (Table 4.6).

Left and right ovarian follicles demonstrated significantly different microbial colonisation patterns (Table 4.7). For left-sided follicles, three bacterial species, *Bacteroides* spp., *Lactobacillus* spp., and *Propionibacterium* spp. had a significant association with colonisation ($p < 0.01$) (Table 4.7). In addition, the presence of *Bifidobacterium* spp., *Candida* spp. and *Staphylococcus* spp. was also significantly associated with colonisation of the left hand side follicle (Table 4.7). In contrast, colonisation of right-sided follicles was significantly ($p < 0.01$) associated with *Actinomyces* spp., *Lactobacillus* spp. and *Propionibacterium* spp. present within follicular fluid aspirated at the time of oocyte retrieval. The regression model revealed no single species was associated with decreased fertilisation rates (results not shown).

Table 4.4 Bacterial load for the most prevalent microbial species isolated from the left and right follicles and the vagina of women with different causes of infertility

		Semi-quantitated CFU / mL in colonised follicles			***Semi-quantitated CFU / mL in contaminated follicles***		
Aetiology	**Most prevalent species**	**Left follicle**	**Right follicle**	**Vagina**	**Left follicle**	**Right follicle**	**Vagina**
Fertile (male factor)	*Staphylococcus* spp.	ND*	ND	$10^3 – 10^5$	ND	10^3	$10^3 – >10^6$
	Streptococcus agalactiae	ND	$10^3 – 10^5$	$10^3 – 10^9$	10^3	10^3	$10^3 – >10^6$
	Actinomyces spp.	10^3 - 10^5	$10^3 – 10^5$	$10^3 – 10^5$	10^3	$10^3 – 10^5$	$10^3 – >10^6$
	Bifidobacterium spp.	ND	10^3	$10^3 – >10^6$	10^3	$10^3 – >10^6$	$10^3 – >10^6$
	Lactobacillus spp.	10^3	10^3	$10^3 – >10^6$	$10^3 – 10^5$	$10^3 – 10^5$	$10^3 – >10^6$
Endometriosis	*Staphylococcus* spp.	10^3	10^3	$10^3 – >10^6$	ND	$10^3 – 10^5$	$10^3 – >10^6$
	Actinomyces spp.	10^3	ND	ND	ND	$10^3 – 10^5$	$10^3 – 10^5$
	Bifidobacterium spp.	ND	ND	$10^3 – >10^6$	10^3	10^3 - $>10^6$	$10^3 – >10^6$
	Lactobacillus spp.	$10^3 – >10^6$	$10^3 – 10^5$	$10^3 – >10^6$	$10^3 – >10^6$	$10^3 – >10^6$	$10^3 – >10^6$
Polycystic ovary syndrome	*Enterococcus faecalis*	ND	10^3	10^3	ND	10^3	$10^3 – 10^5$
	Propionibacterium spp.	10^3	ND	10^3	ND	ND	$10^3 – 10^5$
	Peptostreptococcus spp.	10^3	ND	ND	10^3	ND	ND
	Staphylococcus spp.	$10^3 – 10^5$	10^3	$10^3 – >10^6$	ND	ND	$10^3 – 10^5$
	Streptococcus agalactiae	10^3	ND	10^3	10^3	ND	$10^3 – 10^5$
	Actinomyces spp.	$10^3 – >10^6$	10^3	ND	ND	ND	ND
	Bifidobacterium spp.	10^3	$10^3 – >10^6$	$10^3 – >10^6$	10^3	10^3	$10^3 – >10^6$
	Lactobacillus spp.	$10^3 – >10^6$	10^3	$10^3 – >10^6$	$10^3 – 10^5$	10^3	$10^3 – >10^6$
Genital tract infection	*Escherichia coli*	10^3	$10^3 – >10^6$	$10^3 – >10^6$	ND	10^3	$10^3 – 10^5$
	Staphylococcus spp.	$10^3 – 10^5$	$10^3 – 10^5$	$10^3 – >10^6$	10^3	10^3	$10^3 – >10^6$
	Actinomyces spp.	$10^3 – 10^5$	$10^3 – 10^5$	10^3	$10^3 – >10^6$	$10^3 – 10^5$	$10^3 – 10^5$
	Bifidobacterium spp.	10^3	$10^3 – >10^6$	$10^3 – >10^6$	$10^3 – 10^5$	$10^3 – >10^6$	$10^3 – >10^6$
	Lactobacillus spp.	$10^3 – 10^5$	10^3	$10^3 – >10^6$	$10^3 – 10^5$	10^3	$10^3 – >10^6$
Idiopathic	*Propionibacterium* spp.	$10^3 – 10^5$	$10^3 – 10^5$	10^3	10^3	$10^3 – 10^5$	$10^3 – >10^6$
	Staphylococcus spp.	10^3	10^3	$10^3 – >10^6$	10^3	10^3	$10^3 – >10^6$
	Streptococcus agalactiae	ND	ND	$10^3 – >10^6$	ND	ND	$10^3 – >10^6$
	Bifidobacterium spp.	10^3	10^3	$10^3 – >10^6$	10^3	10^3	$10^3 – >10^6$
	Lactobacillus spp.	$10^3 – 10^5$	10^3	$10^3 – >10^6$	$10^3 – 10^5$	$10^3 – 10^5$	$10^3 – >10^6$

ND = below the limit of detection (10^3 CFU / mL)

The presence of *Lactobacillus* spp. in both right and left follicles was associated with successful embryo transfer ($p < 0.05$) (Table 4.8). *L. gasseri* and *L. crispatus* were detected in both the left and right follicular fluids; however, *L. iners* was detected only in follicular fluids collected from the right ovary (Table 4.13). The presence of *Propionibacterium* spp. and *Streptococcus* spp. in right follicles was associated with poor embryo transfer outcome ($p < 0.05$ and $p < 0.01$ respectively) (Table 4.8). *Propionibacterium* spp. were more frequently isolated from colonised right-sided follicular fluids, whilst *S. agalactiae* was isolated more frequently from the left-sided follicular fluids compared to *S. intermedius*, which was more prevalent in the right follicular fluids (Table 4.13). There was also dependence between pregnancy outcomes and different species in either the left or right ovarian follicular fluid specimens (Table 4.8). For the left ovary, the presence of *Actinomyces* spp., *Bifidobacterium* spp., *Propionibacterium* spp. and *Streptococcus* spp. had a significant association with negative pregnancy outcome ($p < 0.01$). *Lactobacillus* spp. within the left ovary was the only species associated with a positive pregnancy outcome (Table 4.8). For colonised follicular fluid specimens from the right ovary, negative pregnancy outcomes were also associated with *Actinomyces* spp., *Bifidobacterium* spp., and *Streptococcus* spp. ($p < 0.01$), and to a lesser extent *Propionibacterium* spp. and *Staphylococcus* spp. ($p < 0.05$). *S. aureus* and *S. lugdunensis* were both recovered in small numbers from both the left and the right follicular fluids; however, *S. epidermidis* was detected more frequently in follicular fluid collected from the right ovary (Table 4.13). Detection of *Lactobacillus* spp. colonising the right ovary showed a trend towards a positive pregnancy outcome ($p < 0.01$) (Table 4.8).

Table 4.5 A comparison of IVF outcomes for women with different causes of infertility and colonised or contaminated follicular fluid

IVF outcome	Fertilisation rate[1]			Embryo discard rate[2]			Embryo transfer rates[3]			Pregnancy rate[4]		
Aetiology	**Contaminated follicular fluid**	**Colonised follicular fluid**	**p-value**	**Contaminated follicular fluid**	**Colonised follicular fluid**	**p-value**	**Contaminated follicular fluid**	**Colonised follicular fluid**	**p-value**	**Contaminated follicular fluid**	**Colonised follicular fluid**	**p-value**
Endometriosis (n = 49)	182/312 (58%)	122/196 (62%)	>0.05	60/182 (32%)	61/122 (50%)	<0.0001	25/31 (93%)	5/18 (27%)	0.02	16/25 (64%)	0/5 (0%)	0.014
Polycystic ovary syndrome (n = 48)	294/439 (67%)	142/217(65%)	>0.05	91/294 (31%)	23/142 (16%)	>0.05	27/34 (79%)	11/14 (78%)	> 0.05	17/27 (63%)	0/11 (0%)	0.032
Genital infection (n = 39)	203/329 (62%)	109/159 (69%)	>0.05	61/203 (30%)	32/109 (29%)	>0.05	20/28 (71%)	7/11 (64%)	>0.05	10/20 (50%)	1/7 (14%)	>0.05
Male factor[5] (n = 60)	291/520 (56%)	102/179 (57%)	>0.05	131/291 (45%)	32/102 (31%)	0.005	33/44 (75%)	9/16 (56%)	0.002	16/33 (48%)	0/9 (0%)	0.007
Idiopathic (n = 66)	398/616 (64%)	168/242 (69%)	>0.05	160/398 (40%)	52/168 (34%)	>0.05	38/50 (76%)	12/16 (75%)	>0.05	22/38 (58%)	5/12 (42%)	>0.05

[1] No. of oocytes fertilised/total oocytes collected for all women in this cohort, [2] No. of embryos discarded due to developmental arrest or degeneration from the total number of embryos produced by *in vitro* fertilisation, [3] The number of embryo transfers performed on the women within this cohort, not all women had an embryo transfer, [4] The number of pregnancies resulting after embryo transfer, [5] 'Fertile' women control group – women undergoing ART treatment as their male partners are infertile

Table 4.6 Regression analysis of colonisation of follicular fluid for all women with different causes of infertility compared to IVF outcomes

Follicular fluid colonisation	Fertilisation	Embryo transfer	Pregnancy
Endometriosis	NS	†	NS
PCOS	*	†	NS
Genital tract infection	NS	NS	NS
Male factor[1]	NS	NS	NS
Idiopathic	NS	NS	NS
Overall combined colonisation for all aetiologies	NS	*	*

† p = 0.05 - 0.1; * p < 0.05; ** p < 0.01; NS = not significant; [1] Fertile women with infertile male partners; Polycystic ovary syndrome (PCOS)

Table 4.7 Significant bacterial species detected as colonisers of left and right ovarian follicles

Microbial species associated with follicular colonisation	Left follicle colonisation	Right follicle colonisation
Actinomyces species	NS	**
Bacteroides spp.	**	NS
Bifidobacterium spp.	*	NS
Candida spp.	*	NS
Lactobacillus spp.	**	**
Propionibacterium spp.	**	**
Staphylococcus spp.	*	NS
Streptococcus spp.	NS	NS

† p = 0.05 - 0.1; * p < 0.05; ** p < 0.01; NS = not significant

Table 4.8 Regression analysis of ovarian colonisation of the left and right ovaries in relation to adverse or successful IVF outcomes

Microbial species associated with follicular colonisation	Embryo transfer	Pregnancy
Left follicle colonisation	NS	NS
Actinomyces species	NS	**
Bacteroides spp.	NS	NS
Bifidobacterium spp.	NS	**
Candida spp.	NS	NS
I *Lactobacillus* spp.	*	**
Propionibacterium spp.	NS	**
Staphylococcus spp.	NS	NS
Streptococcus spp.	NS	**
Right follicle colonisation	NS	NS
Actinomyces species	NS	**
Bacteroides spp.	NS	NS
Bifidobacterium spp.	NS	**
Candida spp.	NS	NS
I *Lactobacillus* spp.	*	†
Propionibacterium spp.	*	*
Staphylococcus spp.	NS	*
Streptococcus spp.	**	**

† p = 0.05 - 0.1; * p < 0.05; ** p < 0.01; NS = not significant, I = Increased success

4.4.5 Detection of *Mycoplasma* spp. and *Ureaplasma* spp. by real-time PCR of follicular fluid

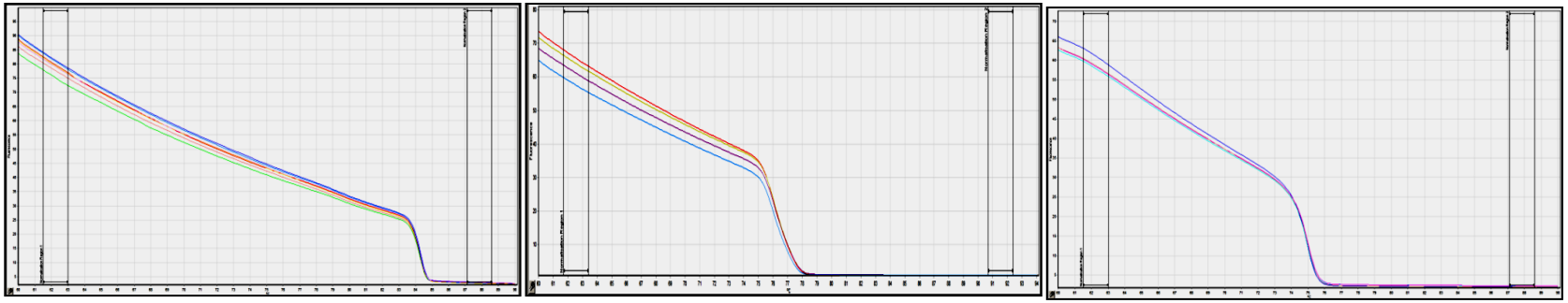
Since *M. genitalium* is difficult to grow in culture or standard culture media, a molecular technique was chosen for the detection of genital mycoplasmas in follicular fluid. Genital mycoplasmas (*M. hominis*, *Ureaplasma* spp.) were detected in 59/462 (12.7%) of follicular fluid specimens tested by real-time PCR assay (Table 4.9). Colonisation/infection by *M. hominis* was most frequent, occurring in 35/462 (7.5%) of follicular fluids identified from women with genital mycoplasmas (Table 4.9). The logistic regression analysis (Table 4.10) demonstrated that infertility caused by endometriosis, polycystic ovary syndrome or genital tract infection was associated with the presence of genital mycoplasmas within the follicular fluid collected at the time of trans-vaginal oocyte retrieval ($p = 0.001 - 0.1$). In the current study, the presence of genital mycoplasmas within follicular fluid had no statistically significant affect on the fertilisation rate, embryo transfer rate or pregnancy rate results not shown).

Table 4.9 Genital mycoplasmas detected within follicular fluid collected at the time of trans-vaginal oocyte retrieval

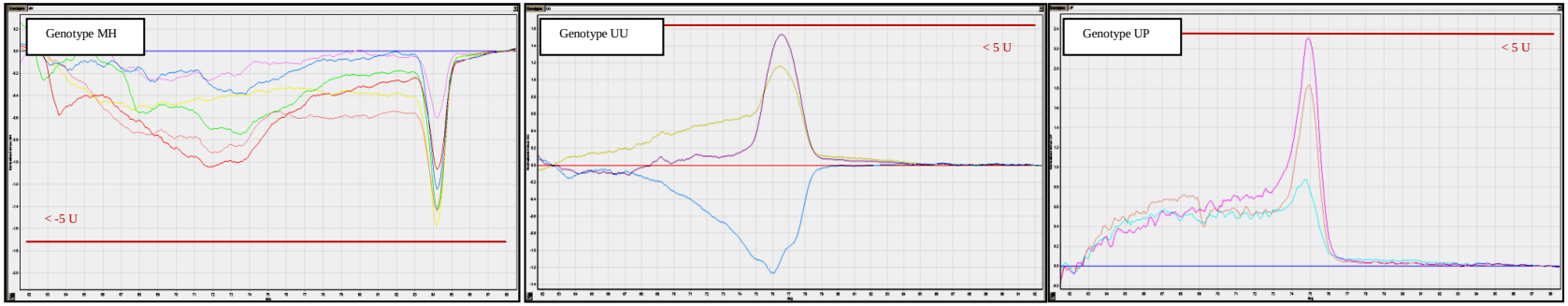
Genital mycoplasma species	Prevalence rate
M. hominis	35/462 (7.5%)
U. urealyticum	14/462 (3.0%)
U. parvum	10/462 (2.1%)
Overall prevalence	59/462 (12.7%)

M. hominis *U. urealyticum* *U. parvum*

a



b



c

No.	C	Name	Genotype	Confidence %
1		MH	MH	100.00
2			MH	94.70
3			MH	91.23
4			MH	94.77
5			MH	99.26
6			MH	97.96
8			MH	90.50

No.	C	Name	Genotype	Confidence %
1		S8	UU	100.00
2			UU	95.78
4			UU	95.79
6			UU	94.49

No.	C	Name	Genotype	Confidence %
12		S3	UP	100.00
13			UP	96.63
14			UP	91.13
15			UP	93.42

Figure 4.2 Real-time PCR analysis and genotyping of genital mycoplasmas in follicular fluid.

Figure 4.2 a HRM was performed for all real-time PCR assays with a ramp of 60 - 90◦ C, rising by increments of 0.1◦ C following a standard melt. The shape of the HRM curve was dependent on the DNA composition of the amplicons generated for each species.

Figure 4.2 b Difference graphs were generated following real-time PCR assay of follicular fluids for detection of genital mycoplasmas. Control DNA was used to normalise the curve for each species. Genotypes were classified as 'same' if they fell within the ≤ 5 U fluorescence difference as demonstrated for the unknown isolates plotted against the *M. hominis* (MH), *U. urealyticum* (UU) and *U. parvum* (UP) control DNA. These graphs show representative positive real-time PCR results for each of these three species.

Figure 4.2 c The Rotor-gene Q software allocated genotypes based on the HRM profile of each unknown isolate. The reference strains for *U. urealyticum* (serovar 8) and *U. parvum* (serovar 3) were used as controls.

Table 4.10 Regression analysis of genital Mycoplasmas and their association with the aetiology of infertility and IVF outcomes

Genital mycoplasma	Aetiology of infertility	p-value
Ureaplasma spp. only	Endometriosis n = 7	**
Ureaplasma spp. only	Genital tract infection n = 6	**
Mycoplasma spp. and *Ureaplasma* spp.	Polycystic ovary syndrome n = 14	**
Mycoplasma spp. and *Ureaplasma* spp.	Genital tract infection n = 8	†

† p = 0.05-0.1;* p < 0.05;** p < 0.01

4.5 Discussion

This study demonstrated that human follicular fluid is often not sterile. Follicular fluid, collected at the time of trans-vaginal oocyte retrieval, is often colonised by microorganisms that are not simultaneously present within the lower genital tract as normal regional flora. This study adopted wound exudates as a model for defining 'colonisation' and 'contamination' because like follicular fluid, wound fluid is also an exudate from plasma. By definition, wound contamination represents the presence of non-replicating microorganisms, whilst in contrast; colonisation represents the presence of replicating microorganisms without an acute host reaction and/or clinical signs or symptoms of infection (Schultz *et al*., 2003; Edwards and Harding, 2004). It should be noted that some samples classified as 'contamination' may actually be colonised samples where the follicular fluids were independently colonised with the same microorganisms as those detected in the vagina. The ability to differentiate between these two cases was beyond the scope and ability of the assays used in this study. For follicular fluids defined as contaminated, many species were present in the follicular fluids only at the lower end of the concentrations reported for those same species isolated from the vagina, which may represent independent colonisation of the follicular fluid by these species rather than direct inoculation of the follicular fluids with vaginal flora at the time of trans-vaginal oocyte retrieval, where a more equivalent concentration would be expected (Table 4.4). As a result of the limitation of the sampling method available in the study, the data presented includes only those specimens that were shown to be 'colonised' for analysis in that cohort. It is interesting that both the concentration and the prevalence of many species including *Propionibacterium* spp., *Bifidobacterium* spp., *S.*

agalactiae and *Staphylococcus* spp. were similar when comparing colonised and contaminated follicular fluids from both the left and the right ovary (Table 4.4).

The isolation of microorganisms from follicular fluid, collected at the time of oocyte retrieval in this current study, is consistent with previous reports documenting asymptomatic contamination of follicular fluid collected as part of an IVF cycle (Cottell *et al.*, 1996). However, this study extends the knowledge in this field by also identifying colonising microorganisms within the follicular fluid. In their study, Cottell *et al*. (1996) analysed the affect of microorganisms from the IVF culture system as a whole by pooling the results obtained for each specimen type (follicular fluid, oocyte retrieval needle washes, semen and culture media) and seeking associations between these results and IVF outcomes and concluded that there were no detrimental effects. However, an independent analysis of their data revealed that bacteria were not isolated from the oocyte retrieval needle wash in 8/15 (53%) of cases, which may mean that the seven bacterial species isolated from the follicular fluid of those 8 women were colonisers of follicular fluid and not contaminants from the vagina, as suggested by the authors. Analysis of the Cottell *et al*. data when the microorganisms were re-classified as 'colonisers' or 'contaminants', based on the definition provided in our study, resulted in a pregnancy rate of 10% if follicular fluid microorganisms were contaminants and only 3% if the microorganisms were defined as colonisers. Cottell *et al*. (1996) reported a significant decrease in the number of oocytes retrieved from women when microorganisms were isolated from their follicular fluid. Taken together, this data suggests, that in contrast to the conclusions of Cottell *et al*. (1996), the presence of microorganisms contaminating

the IVF culture system, and specifically the microorganisms within follicular fluid alone, were associated with adverse IVF outcomes.

Previous studies have also reported the presence of bacteria within porcine follicular fluid. These studies concluded that some bacteria in porcine follicular fluid may inhibit FSH from binding to its receptor on granulosa cells (Sluss *et al.*, 1983, Sluss *et al.*, 1994). In the ovary, the FSH receptor is essential for follicular development and oocyte maturation. Such inhibition would prevent the normal hormonal functioning of FSH. It is therefore plausible that, the presence of microorganisms in human follicular fluid may also result in inhibition of the functioning of FSH and/or damage to the cumulus oocyte complex either by the microorganisms themselves, or the microbial products of metabolism. Therefore one area for future investigation is whether the presence of microorganisms colonising follicular fluid should be investigated as a cause of repeated poor quality cumulus cells or oocytes and possibly poor quality embryos in couples undergoing IVF. Identification of bacteria colonising the follicular fluid in such couples may present the clinician with an opportunity to initiate antimicrobial treatment prior to the next trans-vaginal oocyte retrieval. It may also provide a reason for poor quality oocytes in the absence of another explanation.

In this current study, regardless of whether the women were fertile or infertile, or the cause of infertility, approximately 30% (24 – 37%) of women had colonised follicular fluid and 70% (63 – 76%) had contaminated follicular fluid (Table 4.2). Differences in the microflora of colonised follicular fluid included the isolation of *A. israelii* in colonised follicular fluids from the left ovary, but not from the right

ovarian follicular fluids in these same women. *C. parapsilosis* or *P. granulosum* were only detected as colonisers in the follicular fluids collected from the left or right ovary, they were not also detected in the vagina. *C. albicans, S. viridans, Fusobacterium* spp., and *L. iners* were detected only as a follicular fluid contaminant, but not as a follicular fluid coloniser. Despite previous reports and this current study identifying microorganisms in follicular fluid and the uterus in the absence of symptoms, the current dogma is that the female UGT is sterile (Horne *et al.*, 2008). What is known about the microorganisms at each of these sites is that detectable microorganisms represent a mixed population. Asymptomatic or symptomatic colonisation (commensal microorganisms that are only mildly immunogenic) or infection (acute inflammation to microbial presence) of the uterus and peritoneal cavity has been reported (Spence *et al.*, 1982, Viniker, 1999). If these sites are the source of UGT infection, four mechanisms reported in previous studies could explain how these microorganisms could be transported to the upper genital tract: (1) by ascension of normal regional flora from the vagina to the uterus (Hillier *et al.*, 1993, Holmes *et al.*, 2008); (2) by haematogenous spread of microorganisms from distant sites (Srinivasan *et al.*, 2009); (3) by retrograde infection of the uterus by peritoneal microorganisms gaining access through the uterine tubes; and (4) by direct iatrogenic inoculation (Romero *et al.*, 2001, Sharpe, 2006). Another mechanism of microbial transport to the female upper genital tract may be in seminal fluid or attached to spermatozoa (Gomez *et al.*, 1979, Keith *et al.*, 1984, Svenstrup *et al.*, 2003, Wolner-Hanssen and Mardh, 1984). However, ascending infection and haematogenous dissemination are the routes most frequently implicated in adverse pregnancy outcomes (Romero *et al.*, 2001).

In this study, infertile women with endometriosis had the highest incidence of colonised follicular fluid (37%) when compared to fertile women (27%) (Table 4.2). Culture analyses for these women with endometriosis showed a trend towards significantly increased numbers of follicular fluid colonisers ($p < 0.1$). This may be due to the pathology of endometriosis. Given that reports now confirm that the uterus is at least transiently colonised by microorganisms, it is possible that the source of some of the ovarian microorganisms is the uterus itself. Ovarian endometriomas occur in up to 75 % of women with endometriosis, perhaps suggesting that the uterus itself may be the source of microorganisms in the ovary due to transplantation of colonised endometrial tissue (Vercellini *et al*., 1998, Al-Fozan and Tulandi, 2003, Sznurkowski and Emerich 2008).

The results presented in this chapter suggested that follicular fluid colonised with microorganisms was associated with decreased embryo transfer rates for fertile women ($p = 0.002$, except when colonised by *Lactobacillus* spp., Table 4.8) and for women with infertility resulting from polycystic ovary syndrome ($p = 0.0001$) (Table 4.5). The analysis also found evidence of decreased pregnancy rates for fertile women ($p = 0.007$), as well as women with infertility due to endometriosis ($p = 0.014$) or polycystic ovary syndrome ($p = 0.032$) (Table 4.5), if colonising bacteria were present within follicular fluid.

This study identified a significant difference in the number of microorganisms (as CFU / mL) isolated from the left compared to the right follicular fluids ($p < 0.0001$) (Table 4.3). This is an interesting observation, as similar results were reported by Cottell *et al*. (1996) (42% left and 32% right) and in a murine model of hydrosalpinx

caused by *Chlamydia muridarum* infection of the oviduct (Carey *et al.*, 2009). Such asymmetry in the microbial load may also have implications for IVF. Ovarian endometriomas also occur more frequently in the left ovary (Vercellini *et al.*, 1998, Al-Fozan and Tulandi, 2003, Sznurkowski and Emerich 2008). The successful development of explanted endometrium also relies on extensive vasculature. The blood supply to the ovary may facilitate the haematogenous transport of microorganisms from other anatomical sites. The vasculature to each ovary is asymmetrical and it has been reported that drainage of the left ovary is slower than the right because the left drains into the renal vein and then the inferior vena cava, whilst the right ovary drains directly into the vena cava (Last, 1984). The asymmetrical vascularisation of the ovaries further supports the variation we have identified in the microbial colonisation of left and right follicular fluids. It has been proposed that ovulation occurs more often in the right ovary than the left ovary throughout the reproductive lifetime as a result of the separate, independent vasculature of each ovary (Fukuda *et al.*, 2000, Potashnik, 1987). One study reported significantly better IVF outcomes for the right ovary; the number of oocytes retrieved and the fertilisation rate was increased for oocytes retrieved from the right ovary compared to those from the left ovary (Lan *et al.*, 2010). A greater number of high quality embryos also developed from fertilised oocytes collected from the right ovary. However, the pregnancy and implantation rates were similar for transferred good quality embryos created from either ovary, suggesting that oocyte and early embryo quality are most affected by the *in vivo* follicular environment. Microflora in the follicular fluid, which would potentially persist for a longer time in the left ovary due to the slower regression of the left follicle as a result of the venous drainage (Last, 1984), may contribute to poorer quality oocytes and early embryonic demise.

Knowledge of oocyte laterality may provide a rationale for the selection of embryos for transfer back to the uterus. In IVF, ultrasound studies performed on the ovaries of women undergoing trans-vaginal oocyte retrieval and embryo transfer have confirmed a direct positive relationship between the vascularisation of individual follicles and the subsequent maturity of the oocytes, resulting in increased fertilisation rates and ongoing pregnancy rates (Monteleone *et al.*, 2008).

The findings in this present study also suggest that improved embryo transfer rates are associated with the presence of *Lactobacillus* spp. in both the left and right ovarian follicles (Table 4.8). The significance of the results differed between the left and right ovaries and we propose that this may be because of the presence of different lactobacilli colonising each ovary (*L. crispatus* and *L. gasseri*, left ovary; *L. iners*, right ovary) (Table 4.13). In contrast, the presence of *Propionibacterium* spp. ($p < 0.05$) and *Streptococcus* spp. ($p < 0.01$) within the right ovarian follicles as colonisers was statistically correlated with failed embryo transfers (Table 4.8). IVF pregnancy rates also showed a dependence on the type of microbial colonisation of the ovary. In the left ovarian follicles, colonisation by *Actinomyces* spp. ($p < 0.01$), *Bifidobacterium* spp. ($p < 0.01$), *Propionibacterium* spp. ($p < 0.01$) or *Streptococcus* spp. ($p < 0.01$) was statistically correlated with decreased pregnancy rates. Similar results were obtained for the right ovarian follicles, where the presence of *Actinomyces* spp. ($p < 0.01$), *Bifidobacterium* spp. ($p < 0.01$), *Propionibacterium* spp. ($p < 0.05$), *Staphylococcus* spp. ($p < 0.05$) or *Streptococcus* spp. ($p < 0.05$) was also statistically correlated with decreased pregnancy rates (Table 4.8). The differences in decreased embryo transfer rates or decreased pregnancy rates reflected in the presence of microbial genera as colonisers of follicular fluid may be due to the

effect such microorganisms have on the oocyte quality and early embryonic development. Alternatively, adverse outcomes may be related to the microbial presence and the subsequent immune response within the follicular fluid during folliculogenesis or in the uterus at the time of implantation. Further investigations are required to determine whether these genera affect the embryo quality, leading to failed implantation, early pregnancy loss or pre-term birth.

Just as this study demonstrated differences between the microbial species isolated from the left and right follicular fluids and vagina, previous studies have also reported discordant results when investigating the microorganisms isolated from the lower and upper genital tracts of asymptomatic women. *Bacteroides* spp. and *Streptococcus* spp. have been recovered from peritoneal fluid, but not from the vagina or cervix for 25% of women tested, suggesting that bacteria can exist in the upper genital tract without any evidence of infection (Spence *et al.*, 1982), thus supporting the proposal that the female upper genital tract may be colonised. This study reported the presence of a diverse range of microorganisms, present as colonisers in follicular fluid. Frequently encountered species found to be colonising follicular fluids were members of the normal regional flora of the vagina (*Lactobacillus* spp.), the gastrointestinal tract (*Bifidobacterium* spp., enteric bacteria, *S. agalactiae*), the skin (*Staphylococcus* spp.) and the oral mucosa (*Streptococcus* spp.) (Table 4.13). In an effort to further understand the pathogenesis of inflammatory gynaecological disorders − including endometriosis, endometritis and pelvic inflammatory disease, previous studies have investigated the uterus and/or peritoneal cavity and found that these sites may be transiently colonised by microorganisms (Viniker, 1999). The female upper genital tract, once considered to

be 'normally sterile,' has been shown to harbour many potentially pathogenic microorganisms, even in women without inflammatory disorders (Moller *et al.*, 1985). *Fusobacterium nucleatum*, a periodontal pathogen, rather than the vaginal *Fusobacterium* species, is most frequently recovered from the amniotic fluid of women with amnionitis (Hill, 1993). Therefore, it was proposed that some microorganisms gain access to the female upper genital tract via a haematogenous route. Periodontal pathogens can result in transient bacteraemia, which allows seeding to distant anatomical sites including the uterus and foetal tissues (Daly and Prendiville, 1997). In addition, some species from the vagina or gastrointestinal tract may ascend to the upper genital tract.

In this study, a disparity is species (load) may not be associated with IVF outcomes due to the similar microbial loads (CFU / mL) detected in both the colonised and contaminated follicular fluids of women form each cohort, suggesting that the microbial load is not the most important factor contributing to IVF outcomes. It is likely that similarly to other anatomical sites, the species, load, virulence factors, host resistance and immunological response all play an interdependent role in the overall reproductive outcome. It is becoming increasingly apparent that the microbial load alone is not the only factor to determine whether microorganisms contaminate a body site, colonise a body site or fluid, or proceed to an acute infection (Miller *et al*., 2010). Research now suggests that the individual immune response of each host and the virulence factors of the microorganisms combine to produce the overall outcome. Some women have an altered immune response to challenge by *Candida* spp., which make them more susceptible to infections such as recurrent vulvovaginal candidiasis (Fidel *et al.*, 1997). Alternatively, women with a reported vaginal microflora

composed predominantly of *Atopobium* spp., *Megasphaera* spp., or *Leptotrichia* spp. (bacterial vaginosis associated microorganisms) demonstrated none of the clinical signs of inflammation that would normally accompany such a high microbial load by these species (Verhelst *et al.*, 2005, Witkin *et al.*, 2007).

The results from this current study are in agreement with previous LGT findings (Moore *et al.*, 2000), whereby, in women undergoing IVF treatment cycles, the culture of embryo transfer catheter tips revealed that the presence of hydrogen peroxide producing *Lactobacillus* spp. from the vagina/endometrium were associated with successful IVF pregnancy outcomes. However, if the presence of bacteria other than *Lactobacillus* spp. (including *Escherichia coli, Streptococcus* spp., other *Enterobacteriaceae, Staphylococcus* spp., *Haemophilus* spp. and mixed cultures) was detected from culture of the embryo transfer catheter tip, all of which can be part of the normal regional flora of the lower genital tract, then these were associated with reduced number of successful IVF pregnancies and an increase of early miscarriage rates (Eckert *et al.*, 2003, Egbase *et al.*, 1996, 1999, Moore *et al.*, 2000). Furthermore, IVF pregnancy rates per embryo transfer were also lower for patients with culture positive catheter tips (24.1%) compared to patients with culture negative catheter tips (33.3%) (Fanchin *et al.*, 1998). Bacterial vaginosis, a condition in which the *Lactobacillus* spp. dominant flora within the vagina is replaced by other flora including Gram-negative *Gardnerella* spp. and *Mobiluncus* spp., has been associated with increased pregnancy losses in both natural (Hay *et al.*, 2000) and IVF pregnancies (Ralph *et al.*, 1999). In these studies of women undergoing embryo transfers, the microorganisms recovered from the embryo-transfer catheter tip were reported as cervico-vaginal flora. However, no microbiological specimens from the

upper genital tract were collected and/or screened. It may therefore be possible that the microorganisms detected on the embryo transfer catheter tip originated from the follicular fluid.

The role of microorganisms in adverse pregnancy outcomes, including miscarriage, stillbirth, pre-term rupture of membranes and pre-term birth is well established. Previous studies have reported that the microorganisms isolated from women with intact membranes, who undergo pre-term labour, include: *Ureaplasma* spp., *M. hominis*, *Gardnerella vaginalis*, *Peptostreptococcus* spp. and *Bacteroides* spp. (Andrews *et al.*, 1995, Gibbs *et al.*, 1992, Hauth and Andrews, 1998, Hillier *et al.*, 1988, Krohn *et al.*, 1995); whilst in cases of premature membrane rupture, the species most frequently recovered include *Streptococcus agalactiae* and *E. coli* (Goldenberg *et al.*, 2000). Alterations in the female lower genital tract flora, resulting in bacterial vaginosis have also been associated with asymptomatic plasma cell endometritis (Korn *et al.*, 1995) and an increased risk of spontaneous pre-term labour (Eschenbach *et al.*, 1984, Gravett *et al.*, 1986, Hillier *et al.*, 1995, Silver *et al.*, 1989). The follicular fluid is a previously un-investigated source of microorganisms. These microorganisms have the potential to affect early pregnancy events, or may be sources of late adverse pregnancy events.

This current study also found that the presence of *Ureaplasma* spp. only, or the combination of *Mycoplasma* spp. and *Ureaplasma* spp. within follicular fluid, was significantly associated with infertility due to endometriosis or genital tract infection ($p = 0.01 – 0.001$), whilst infertility due to polycystic ovary syndrome was

significantly associated with the presence of both *Mycoplasma* spp. and *Ureaplasma* spp. within follicular fluid ($p = 0.01 – 0.001$).

The data from this study demonstrate several novel concepts: (1) follicular fluid is not sterile, but in fact contains a diverse range of microorganisms at varying concentrations; (2) follicular fluid can be colonised or contaminated by microorganisms; (3) follicular fluid colonisation or contamination is not related to the aetiology of infertility; (4) species diversity is higher in the vagina than in follicular fluid; (5) microbial load does not appear to be the most significant factor in IVF outcomes; (6) *Lactobacillus* spp. within follicular fluid was correlated with positive IVF outcomes; (7) some species isolated from follicular fluid appear to be opportunistic pathogens (including *Propionibacterium* spp., *Streptococcus* spp. *Actinomyces* spp., *Staphylococcus* spp. and *Bifidobacterium* spp.) and were related to adverse IVF outcomes including decreased embryo transfer rates, which may reflect poor quality embryos due to damage by microorganisms or their metabolites and decreased pregnancy rates or no pregnancy (failed implantation) (Tables 4.11 and 4.12).

In summary, the presence of microorganisms in follicular fluid may be a significant contributor to adverse IVF outcomes. It has been shown that ovarian follicles can be colonised independently from the normal regional flora or infections of the lower reproductive tract, suggesting that even asymptomatic women with repeated failed IVF cycles should be screened for the presence of microorganisms in follicular fluid and possibly treated with antimicrobials in order to increase treatment success rates.

Table 4.11 Summary of colonised follicular fluid and IVF outcomes

Colonised follicular fluid	IVF outcomes	Aetiology	p-value	Reference
Embryo transfer	Decreased embryo transfer rates	Fertile women Endometriosis	0.002 0.02	Table 4.5
Frozen embryos	Increased embryo discard rates	Endometriosis	< 0.0001	Table 4.5
Pregnancy	Decreased pregnancy rates	Fertile women Polycystic ovary syndrome Endometriosis	0.007 0.032 0.014	Table 4.5

Table 4.12 Summary of the logistic regression analysis of colonised left and right follicular fluids and associated successful and adverse IVF outcomes

Colonised follicular fluid	IVF outcomes	Species implicated	p-value	Reference
Colonisation left ovarian follicles		*Lactobacillus* spp. *Bacteroides* spp. *Propionibacterium* spp. *Bifidobacterium* spp. *Staphylococcus* spp. *Candida* spp.	<0.01 <0.01 <0.01 <0.05 <0.05 <0.05	Table 4.8
Embryo transfer	Increased embryo transfer rates	*Lactobacillus* spp.	<0.05	Table 4.8
Pregnancy	Decreased pregnancy rates	*Actinomyces* spp. *Propionibacterium* spp. *Bifidobacterium* spp. *Streptococcus* spp.	<0.01 <0.01 <0.01 <0.05	Table 4.8
Colonisation right ovarian follicles		*Lactobacillus* spp. *Actinomyces* spp. *Propionibacterium* spp.	<0.01 <0.01 <0.01	Table 4.8
Embryo transfer	Increased embryo transfer rates	*Lactobacillus* spp.	<0.05	Table 4.8
Embryo transfer	Decreased embryo transfer rates	*Streptococcus* spp. *Propionibacterium* spp.	<0.01 <0.05	Table 4.8
Pregnancy	Decreased pregnancy rates	*Actinomyces* spp. *Bifidobacterium* spp. *Propionibacterium* spp. *Staphylococcus spp* *Streptococcus* spp.	<0.01 <0.01 <0.05 <0.05 <0.05	Table 4.8

Table 4.13 Microorganisms detected in the follicular fluid and vaginal secretions of women with various causes of infertility

	Colonised			**Contaminated**			**Male factor (fertile women)**			**Endometriosis**			**Polycystic ovary syndrome**			**Genital infection**			**Idiopathic**		
Microbial species	Left Ovary (L)	Right Ovary (R)	Vagina (V)	Left	Right	Vagina	L	R	V	L	R	V	L	R	V	L	R	V	L	R	V
Actinomyces species	A +	+	+	+	+	+	35	35	5	-	-	-	-	-	-	28	14	5	61	48	-
	B						31	26	5	-	-	-	-	-	-	16	9	4	15	13	-
A. israelii	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	3	-	4	-	-	-
							-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A. meyeri	+	-	+	-	-	+	-	-	1	-	-	-	-	-	-	-	-	-	-	-	2
							-	-	1	-	-	-	-	-	-	2	-	-	-	-	2

+ present, - absent; A (top line of each species) present as 'contaminants', B (bottom line of each species) present as 'colonisers', numbers represent the number of women each species was detected in for left and right follicular fluids and vaginal secretions.

A. naeslundii	+	-	+	+	+	+	4	2	8	-	-	16	-	-	-	-	-	6	-	4	-
							-	-	8	2	-	4	-	-	-	-	-	6	-	-	-
Bacteroides spp.	-	+	+	-	+	+	-	-	-	-	5	-	-	3	-	-	12	-	-	-	7
							-	1	-	-	3	-	-	-	-	-	3	-	-	-	1
Bacteroides fragilis	+	+	+	+	+	+	1	3	-	-	6	-	-	-	6	-	6	6	22	-	-
							-	-	-	-	3	-	-	-	-	1	3	3	3	-	-
Bifidobacterium spp. 1	+	+	+	+	+	+	26	14	52	1	1	58	28	13	32	7	21	15	12	7	83
							-	5	19	-	-	41	12	-	10	4	15	18	14	8	12
Bifidobacterium spp. 2	-	+	+	-	+	+	-	-	17	-	1	-	-	-	51	-	1	19	-	1	61
							-	-	12	-	-	-	-	-	33	-	3	7	-	-	8
Candida albicans	+	+	+	-	+	+	-	-	35	-	-	24	-	-	12	-	12	10	-	-	-
							-	-	10	-	-	6	1	-	-	-	8	-	-	-	-
C. glabrata	+	+	+	+	+	+	-	-	7	-	8	12	-	-	6	-	-	15	22	6	64
							-	-	2	-	-	10	-	-	-	-	-	4	3	7	8
C. parapsilosis	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-	1	4	-	-	-	5
							-	-	-	11	-	-	-	-	-	-	3	-	-	-	-

Clostridium butyricum	-	-	+	+	-	+	-	-	15	-	-	-	-	-	2	-	-	5	2	-	29
							-	-	-	-	-	-	-	-	1	-	-	5	-	-	10
Clostridium spp.	-	-	+	+	-	+	1	-	-	-	-	-	-	-	2	-	-	-	-	-	-
							-	-	-	-	-	-	-	-	4	-	-	-	-	-	-
C. ramosum	-	-	+	-	-	+	-	-	4	-	-	-	-	-	-	-	-	9	-	-	-
							-	-	4	-	-	-	-	-	-	-	-	-	-	-	-
Corynebacterium spp.	-	+	+	-	-	+	-	-	8	-	-	-	-	-	10	-	-	5	-	-	-
							-	-	5	-	-	-	-	1	2	-	-	13	-	-	-
C. auromucosum	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3
							-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Escherichia coli	+	+	+	-	+	+	-	-	15	-	-	-	-	-	7	-	6	21	-	1	22
							-	-	4	-	-	11	-	-	2	1	4	7	-	-	3
Enterococcus faecalis	-	+	+	-	-	+	-	-	6	-	-	10	-	-	11	-	-	7	-	-	-
							-	-	6	-	-	-	-	24	10	-	-	2	-	-	-

Egghertella lenta	-	-	+	-	-	+	-	-	4	-	-	-	-	-	2	-	-	8	-	-	12
							-	-	1	-	-	-	-	-	1	-	-	-	-	-	-
Fusobacterium spp.	-	-	-	+	-	+	1	-	-	-	-	10	-	-	-	-	-	-	-	-	-
							-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gemella spp.	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	17	-	-	3
							-	-	-	-	-	-	-	-	-	-	-	5	-	-	-
Klebsiella spp.	-	-	+	-	-	+	-	-	-	-	-	-	-	-	2	-	-	1	-	-	-
							-	-	-	-	-	-	-	-	1	-	-	2	-	-	-
Lactobacillus spp.	+	+	+	+	+	+	16	12	67	7	16	81	32	13	65	8	5	42	66	33	125
							31	11	37	6	7	36	25	1	7	4	3	15	13	13	9
L. crispatus	+	+	+	+	+	+	18	5	51	19	3	24	4	-	31	20	7	52	1	-	70
							10	5	21	12	10	4	1	-	30	13	4	5	-	-	7
L. gasseri	+	-	+	+	+	+	3	-	23	2	2	3	1	-	9	3	-	9	-	-	15
							-	-	2	6	-	3	-	-	1	1	-	-	-	-	2
L. iners	-	-	-	-	+	-	-	-	-	-	-	-	-	3	-	-	-	-	-	-	-
							-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

L. jensenii	+	+	+	+	-	+	14	-	42	9	-	17	-	-	29	3	-	14	4	-	24
							-	-	-	11	10	3	-	-	1	1	2	1	-	-	4
Propionibacterium spp.	+	-	-	-	-	+	-	-	16	-	-	-	-	-	-	-	-	-	-	-	-
							-	-	-	-	-	-	1	-	-	-	-	-	-	-	-
P. acnes	+	+	+	+	+	+	22	33	35	-	-	2	-	-	3	-	-	2	60	85	54
							21	31	13	11	-	3	-	-	-	1	1	2	13	25	2
P. avidum	-	-	+	-	-	+	-	-	10	-	-	-	-	-	9	-	-	-	-	-	8
							-	-	1	-	-	-	-	-	-	-	-	-	-	-	-
P. granulosum	+	+	-	+	+	+	1	1	4	-	-	-	-	-	-	-	-	10	-	-	11
							5	5	-	-	-	-	-	-	-	-	-	-	-	-	-
P. propionicus	+	+	+	+	-	+	-	-	6	-	-	2	-	-	2	-	-	-	6	-	-
							-	-	3	-	-	3	-	-	4	1	-	-	7	2	-
Prevotella disiens	-	-	+	-	-	+	-	-	5	-	-	-	-	-	-	-	-	15	-	-	-
							-	-	1	-	-	-	-	-	-	-	-	-	-	-	-

P. melanogenicus	-	-	+	-	-	+	-	-	4	-	-	-	-	-	-	-	-	2	-	-	4
							-	-	1	-	-	-	-	-	-	-	-	3	-	-	2
Peptinophilus	+	-	+	+	+	+	1	-	10	-	-	-	-	-	2	-	6	-	-	-	-
asaccharolyticus							-	-	3	-	-	-	-	-	4	1	-	-	-	-	-
Peptostreptococcus spp.	+	+	+	+	+	+	2	-	1	-	-	-	13	-	-	-	-	-	-	22	-
							-	-	1	-	-	-	1	-	-	1	5	2	-	3	-
Staphylococcus spp.	+	+	+	-	+	+	-	-	18	-	2	6	-	-	15	-	-	15	-	7	17
							-	-	6	11	10	10	-	-	3	1	-	12	-	-	1
S. aureus	+	-	+	+	+	+	-	-	29	-	-	30	-	-	3	1	1	21	22	-	50
							-	-	10	-	-	15	-	-	3	1	-	8	-	-	7
S. epidermidis	+	+	+	+	+	+	-	2	41	-	-	36	-	-	-	3	9	3	6	6	32
							-	-	11	-	-	24	-	-	-	1	6	2	7	7	6
S. lugdunensis	+	-	+	+	+	+	-	-	22	-	-	28	-	-	8	6	7	6	-	1	8
							-	-	8	-	-	13	-	-	4	4	4	3	-	-	2

Streptococcus spp.	+	+	-	+	+	+	-	-	4	-	-	-	-	-	6	6	6	-	-	-	-
							-	-	-	-	-	-	-	-	-	5	4	-	-	-	-
S. agalactiae	+	+	+	+	+	+	10	1	31	-	-	52	15	-	20	1	5	17	-	-	34
							-	-	16	-	-	38	13	-	15	-	3	9	-	-	8
S. intermedius	+	+	+	+	+	+	1	1	39	-	-	12	-	-	15	-	2	9	-	6	11
							-	-	12	-	-	10	-	-	3	-	-	3	1	7	6
S. viridans	-	-	-	+	-	+	2	-	-	-	-	-	-	-	-	-	-	-	-	-	15
							-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Veilonella spp.	-	+	+	+	+	+	1	-	1	-	-	-	-	15	2	-	-	3	-	-	-
							-	-	1	-	-	-	-	12	1	-	-	-	-	7	-
Unknown A	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	2	-	-	5
							-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Unknown B	-	-	+	-	-	+	-	-	2	-	-	-	-	-	7	-	-	-	-	-	-
							-	-	-	-	-	-	-	-	2	-	-	-	-	-	-
Total number of isolates							**257**	**193**	**862**	**108**	**87**	**657**	**147**	**85**	**511**	**149**	**204**	**516**	**360**	**319**	**874**

CHAPTER FIVE

IN VITRO CHARACTERISATION OF BIOFILM FORMATION IN FOLLICULAR FLUID

5.1 Introduction

Follicular fluid collected at the time of trans-vaginal oocyte retrieval does not appear turbid, as would be expected if there were an acute infection, and microorganisms and resultant immune cells were present. This discrepancy prompted an investigation of follicular fluid specimens to determine if the microorganisms present may form biofilms *in vitro* and thus persist as a reservoir of bacterial cells for an extended period without causing symptoms of infection. In addition, the follicular fluid from women undergoing IVF cycles contains high levels of the steroid hormones, oestradiol and progesterone, which may influence the growth of microorganisms. The persistence of microorganisms within the ovarian follicle and within follicular fluid may be a reason for poor IVF pregnancy outcomes.

Biofilms are ubiquitous, they play a major role in pathogenicity and their formation within the genital tract has been reported in cases of infection and colonisation (Patterson *et al.*, 2009, Pruthi *et al.*, 2003, Romero *et al.*, 2008). Research characterising microorganisms in human infections suggested that bacteria undergoing rapid replication, frequently cause an acute infection, whilst a biofilm-growth strategy would evade the host immune response and result in asymptomatic infection/colonisation (Zegans *et al.*, 2002). Within a biofilm, replication rates were less rapid, when compared to planktonic cell growth. Biofilms persisted for extended periods whilst continuing to shed low numbers of bacterial cells and metabolites, which were capable of priming the host immune response but this, did not result in the eradication of the infectious cells (Zegans *et al*., 2002).

The focus of this chapter was to investigate *in vitro* if: (1) the growth of bacterial species (isolated from human follicular fluid) was influenced by the presence of steroid hormones, oestradiol and progesterone; (2) the bacteria present within follicular fluid were capable of growth over time and; (3) these same bacteria may form biofilms *in vitro*.

5.2 Materials and Methods

5.2.1 Experimental design

In this study, a sub-cohort of 36 follicular fluids from the main cohort was analysed. This cohort was randomly selected from the follicular fluid specimens with a sufficient volume of fluid remaining for testing. Follicular fluids were also selected to include an even mix of clear and blood-stained fluids. Follicular fluids collected from only 13 women were of a sufficient volume for all the analyses described to be performed. For 11 of these women, follicular fluid collected from both the left and right ovary was cultured individually (results not shown). In the previous chapter, standard microbiological culture media were used, however in this chapter; steroid hormones, oestradiol and progesterone were added to all culture media prior to inoculation with follicular fluid. The sub-cohort was then analysed for the *in vitro* growth characteristics of the microflora over time and in the presence of the added exogenous hormones. Details of the hormones are described in section 5.2.9.

5.2.2 Specimen collection

Specimen collection was performed as described in Section 3.1.

5.2.3 Follicular fluid culture and colony identification

Follicular fluid specimens were cultured as previously described (Section 3.2).

5.2.4 16S rRNA PCR and sequencing

Identification of bacteria isolated from clinical specimens was performed as described in Sections 3.3 - 3.6.

5.2.5 Biofilm assay

Twenty-four follicular fluid specimens were thawed; 300 µL of each fluid was added to a single sterile 14 mm coverslip in one well of a 24-well microtitre plate for the biofilm assay. Triplicate microtitre plates were prepared, which were then incubated in aerobic and anaerobic conditions at 37° C: two coverslips were stained as described below, and the bacteria from the third was inoculated onto media as described in section 5.2.5. These follicular fluid specimens were incubated within the microtitre plates undisturbed for a ten-day period (the average period of development of follicular fluid within a maturing ovarian follicle) (Speroff and Fritz, 2005). Upon removal, coverslips were gently rinsed with PBS to remove any unbound cells. The coverslips were placed on clean microscope slides, and processed using a modified method of Allison and Sutherland (Allison, 1984). Briefly, coverslips were covered with 10 mM cetyl pyridinium chloride and air dried before heat fixation. The biofilms present then were stained for 15 minutes with a 2:1 mixture of saturated Congo red (Sigma Aldrich, Castle Hill, NSW) solution and 10% Tween 20 (Sigma Aldrich). Slides were then rinsed, counterstained with 10% Ziehl carbol fuchsin, rinsed again and dried at 37° C. The prepared coverslips were viewed by light and confocal microscopy.

Light microscopy was performed using an Olympus BX41 camera and microscope (Olympus, North Ryde, NSW). Images were captured with a Micro Publisher 3.3 RTV camera (Adept Electronic Service, Warriewood, NSW) and QCapture Pro

software (QImaging, Surrey, BC). Biofilms were examined under total magnifications of × 100 and × 400.

Laser scanning confocal visualisation was performed using a Leica SP5 microscope (Leica Microsystems, North Ryde, NSW) with a × 10 objective lens, a × 63 magnification oil immersion lens, a numerical aperture of 1.4, and laser excitation was at 561 nm capturing the spectral range 567-668 nm. A series of z stack scans were performed through a section (2 – 62 µm range) and the series of images were compiled using a maximum projection at a total magnification of × 630, or × 630 with an additional × 4 zoom.

Biofilm maturity was graded based on the presence of key characteristic and structural features observed (Simmons *et al.*, 2007): grade I-planktonic cells (isolated free floating cells, not adherent to the slide) and cells adherent to the conditioning film; grade II-microcolonies, and groups of cells (most likely planktonic) attached to each other; grade III-extending/growing microcolonies (towers) with extracellular matrix fibrils creating interconnections between the microcolonies giving a cobweb appearance; grade IV-towers with subterranean channels and amorphous extracellular material giving a honeycomb appearance between the microcolonies.

5.2.6 Biofilm culture

The third coverslip was rinsed with sterile PBS and a sterile swab was used to remove the biofilm from the surface of the coverslip. A bacterial suspension then was made in thioglycollate broth; a 1 µL calibrated inoculating loop was used to subculture the biofilm onto horse blood agar and anaerobic blood agar plates

(Oxoid). These were incubated aerobically and anaerobically at 37° C. Colony identification was performed as previously described in the General Methods Chapter (Section 3.2).

5.2.7 Bacterial growth on glass beads for biofilm quantification

Further aliquots (300 µL) of each of the 24 follicular fluid specimens were added to wells of a microtitre plate that contained sterile pre-weighed glass beads. Duplicate plates were incubated aerobically or anaerobically for 10 days at 37° C. Biofilm mass was calculated after removing the beads from the follicular fluid and weighing the beads (Giridhar *et al.*, 1994).

5.2.8 Wall adherence assays

A 1 mL aliquot of each of the 24 follicular fluid specimens was incubated at 37° C for a period of 10 days in a sterile cryovial. After 10 days, the follicular fluid was removed and 1.5 mL of safranin O stain was added for 15 minutes, the stain was decanted and 1.5 mL of sterile PBS was added (Christensen, 1982). After vortexing, the absorbance at 500 nm was read in a spectrophotometer against a PBS control.

5.2.9 Culture of (aliquots of) follicular fluid

The 24 follicular fluid specimens were also aliquotted aseptically into 1.7 mL microcentrifuge tubes (1 mL of follicular fluid) and incubated at 37° C aerobically. These specimens were vortexed and subcultured daily, using a 1 µL calibrated inoculating loop, onto horse blood agar and anaerobic blood agar plates (Oxoid) and incubated aerobically and anaerobically at 37° C. After incubation, the number of CFU / mL of follicular fluid were semi-quantified. The 1 µL calibrated inoculating loop carries approximately 10^{-3} mL of follicular fluid, so the number of colonies on

the plate can be used to determine the approximate number of CFU / mL of follicular fluid by multiplying by 10^3.

5.2.10 Hormonal effect on bacterial growth in follicular fluid

An additional 12 frozen follicular fluid aliquots were thawed and cultured on agar plates and thioglycollate broths containing oestradiol and progesterone (Sigma-Aldrich) at concentrations of 375 µg / L and 800 µg / L respectively. These are the median concentrations reported in the follicular fluid collected from hyperstimulated women undergoing trans-vaginal oocyte retrieval (Kushnir *et al.*, 2009, Loret de Mola, 1999), Follicular fluid specimens were also cultured on hormone-free media, using 1 µL of each of the 12 specimens to evaluate the effect of the presence or absence of hormones on bacterial growth. Hormones were only used in combination, not individually, as both are present within follicular fluid at high concentrations at the time of trans-vaginal oocyte retrieval. After seven days, positive thioglycollate broths were vortexed and a sterile 1 µL calibrated loop was used to subculture broth onto blood agar for quantification and identification of the bacteria present, as previously described in section 3.2.

5.3 Results

5.3.1 Follicular fluid culture and bacterial colony identification

Bacteria were cultured from each of the 24 follicular fluids. A single bacterial species was isolated from 15/24 (63%) of the specimens, of which 7/15 (47%) contained *Lactobacillus* species. *Propionibacterium* spp. (from 5 samples), *Peptostreptococcus* spp. (n = 2), or *Salmonella entericus* (n = 1) were isolated in the remaining specimens. Two bacterial species were isolated from 6/24 (25%)

specimens, of which five (83%) contained *Lactobacillus* spp. Only 3/24 (12%) follicular fluid specimens contained three bacterial species, and each of these specimens contained a *Lactobacillus* spp. The *Lactobacillus* spp. and *Propionibacterium* spp. were the most prevalent isolates detected in follicular fluid, isolated from 51% and 14% specimens respectively (Table 5.1).

5.3.2 Biofilm well culture – 75% of follicular fluid isolates formed biofilms *in vitro*

Each of the 24 follicular fluid specimens was tested for biofilm formation. Biofilms formed in 18/24 (75%) of follicular fluids after incubation at 37° C. Of these, 14 were monomicrobial and 4 were polymicrobial. The bacterial species *L. gasseri, L. crispatus, Bifidobacterium longum*, *S. agalactiae, S. anginosus* and *S. entericus*, if initially present in follicular fluid, were always recovered from the biofilm well sub-cultures. Other bacterial species, including CoNS, *Peptostreptococcus* spp. and *E. coli*, were only cultured from the original follicular fluid specimen. In 9/24 (37%) of the follicular fluids tested, the primary culture demonstrated polymicrobial colonisation, but only a single species was detected after 10 days of incubation within the microtitre plate. None of the wells became contaminated with ‘new ‘ species as a result of poor aseptic technique; if only a single species was detected in the primary follicular fluid culture, the same single species was always detected in the well. Bacterial species were isolated following incubation under both aerobic and anaerobic conditions if the species was a facultative anaerobe (*Staphylococcus* spp., *Streptococcus* spp. and *Lactobacillus* spp.). For strict anaerobes (*Bifidobacterium* spp., *Peptostreptococcus* spp., the species were isolated only when the follicular fluid specimens were incubated anaerobically.

Table 5.1 Bacterial genera isolated and identified from cultures of follicular fluid

Genus and species	Follicular fluid culture n = 24	Percentage of total n = 35 isolates	Biofilm culture n = 18	Percentage of total n = 23 isolates
Lactobacillus gasseri[1]	9	26%	9	41%
L. crispatus[1]	7	20%	7	31%
Propionibacterium spp.	5	14%	1	5%
CoNS[2]	3	9%	0	0%
L. jensenii[1]	2	5%	1	5%
Peptostreptococcus spp.	2	5%	0	0%
B. longum	2	5%	1	5%
S. agalactiae	1	3%	1	5%
S. anginosus	1	3%	1	5%
Micrococcus spp.	1	3%	1	5%
S. entericus	1	3%	1	5%
E. coli	1	3%	0	0%
Total number of isolates	35		23	

[1] *Lactobacillus* spp. were the most prevalent bacteria in follicular fluid 18/35 (51%), [2]CoNS coagulase negative staphylococci

5.3.3 Biofilm assay – images

The images are representative (Figure 5.1) of the different types of biofilms observed, which were graded as grade IV biofilms 7/18 (39%), grade III biofilms 7/18 (39%) and grade II biofilms 4/18 (22%). Each grade was observed for follicular fluids incubated both under aerobic and anaerobic atmospheric conditions.

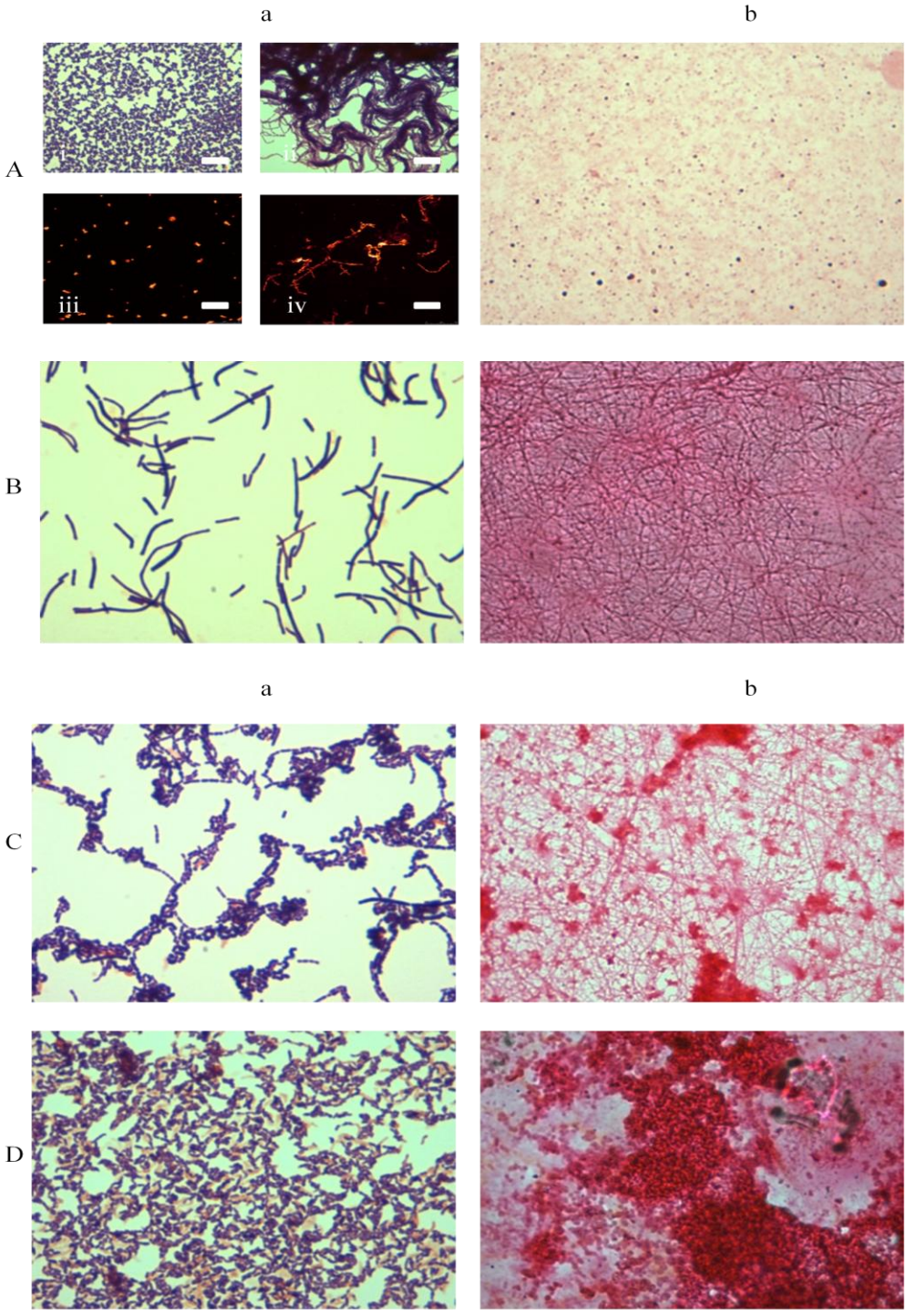


Figure 5.1 Colour images of bacteria and biofilms. Figure 5.1 A (a) (i, ii) B – D (a)) Light microscopy image at × 1000 total magnification of Gram-stained bacteria *Lactobacillus* spp. and *S. agalactiae* cultured from biofilms . (A (a)) (iii, iv) Laser scanning confocal microscopy image of these individual bacterial cells at × 630 total magnifications plus × 4 zoom, captured in Glow. (B - D (a)) Gram stains of the bacterial cells that formed the biofilms. (A - D (b)) Light microscopy image at × 1000 total magnification of Congo red stained *Lactobacillus* spp. biofilms grown for 10 days on glass coverslips.

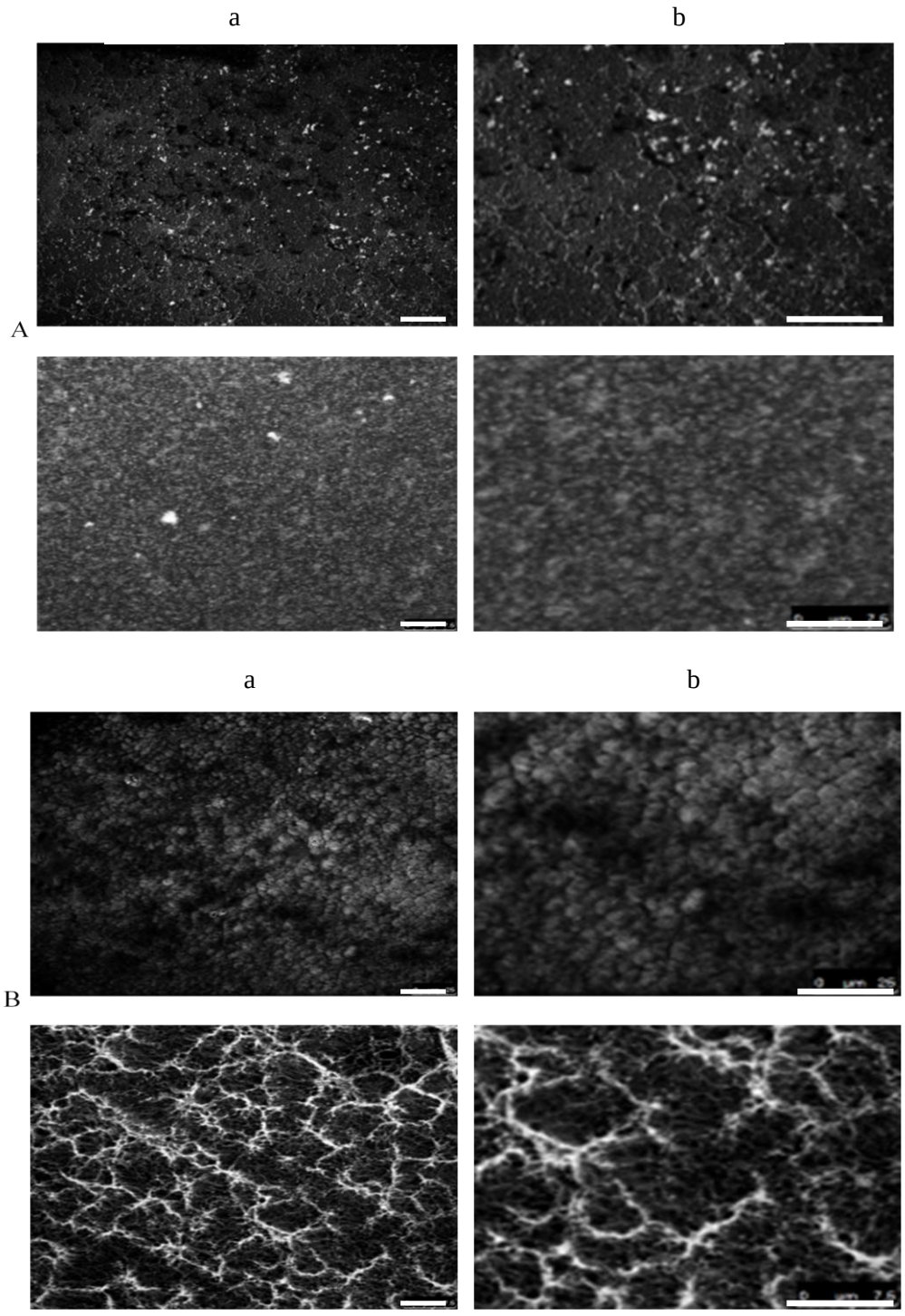
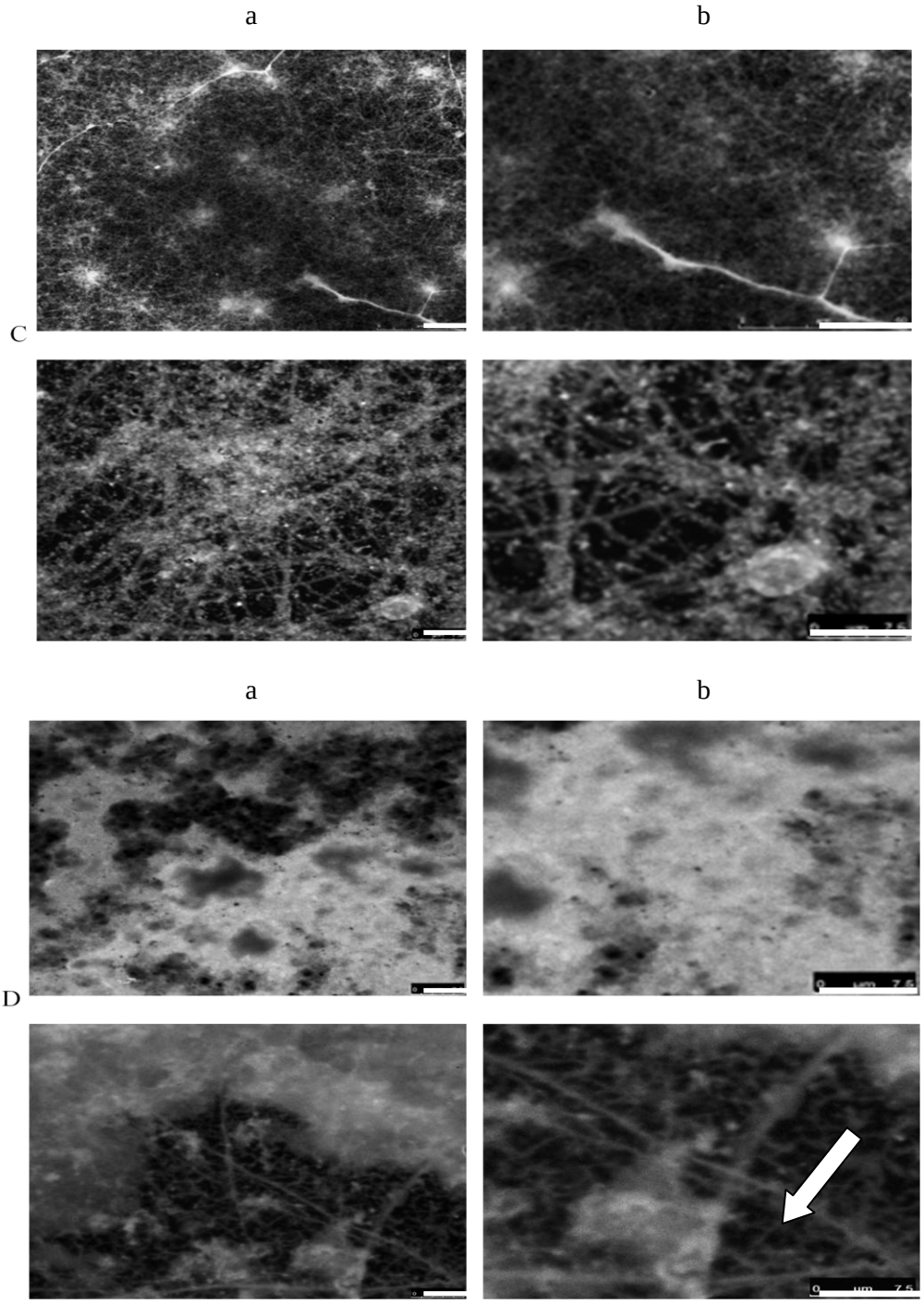


Figure 5.2 Black and white laser scanning confocal microscopy images of biofilms.
Figure 5.2 (A – D (a)) Laser scanning confocal microscopy image of various grades of biofilms at × 630 total magnification. Scale bars represent 25 µM for all 'a' images. (A – D (b)) Laser scanning confocal microscopy images of various grades of biofilms at × 630 total magnifications plus × 4 zoom. (C (a and b)) Image of cob-webbing. (D (a and b)) Image of the honeycombed region. The arrow points to the cavities in the honeycombs. Grade II biofilms presented in Figure 5.2 A (a and b) demonstrated few microcolonies, some of which extended to form towers. A grade III biofilm was characterised by a carpet-pile appearance with some microcolonies extending to form towers (Figure 5.2 B (b)). Grade IV biofilms had a honeycomb appearance where the continuous layers of cells covered the coverslip with

clusters forming prominences (Figure 5.2 D (a) and (b)). Scale bars represent 7.5 µM for all 'b' images.



The simplest of the biofilms visualised by light microscopy was seen for follicular fluid cultures that were incubated aerobically. It can be observed that grade II

biofilms presented in Figure 5.2 A (b) demonstrated few microcolonies, some of which extended to form towers (outward growing masses of bacterial cells). Initial microcolonies are formed by cells, which proliferate at fixed positions (Klausen *et al.*, 2003a,b).Towers appeared white in greyscale using confocal microscopy, as shown in Figure 5.2 A (a) and A (b). A grade III biofilm was characterised by fibril formation, as seen by light microscopy following anaerobic incubation (Figure 5.1 B (b)), and a carpet-pile appearance with some microcolonies extending to form towers (confocal microscopy) as shown in Figure 5.2 B (a) The carpet-pile may be representative of the cap and stalk populations previously described in biofilms (Pamp *et al.*, 2008). The extracellular matrix appeared to be more abundant in microcolonies. When magnified (× 4), crater-like formations were visible (Figure 5.2 B (b)). Prior studies have described local biofilm dispersion as a hollowing-out of microcolonies, once a break point was reached (Pamp *et al.*, 2008, 2009, Sanchez *et al.*, 2011) . In the grade IV biofilm grown under anaerobic conditions (Figure 5.1 C (b)), complex networks were observed between the microcolonies and towers and these had a cobwebbed appearance, as shown in Figure 5.2 C (a) and (b). Grade IV biofilms were also visualised following anaerobic incubation (Figure 5.1 D (b)) and an amorphous extracellular matrix was observed surrounding the interconnecting microcolonies, towers and cells. In these biofilms, continuous layers of cells covered the coverslip with clusters forming prominences. These biofilms had a honeycomb appearance (Figure 5.2 D (c) and (d)). The biofilms can also be visualised using three dimensional and orthogonal confocal images. This image (Figure 5.3) demonstrated the presence of channels within the biofilms and hollow areas under the towers. All observed biofilms showed an uneven spatial distribution, a previously described characteristic of *in vitro* biofilms (Sanchez *et al.*, 2011).

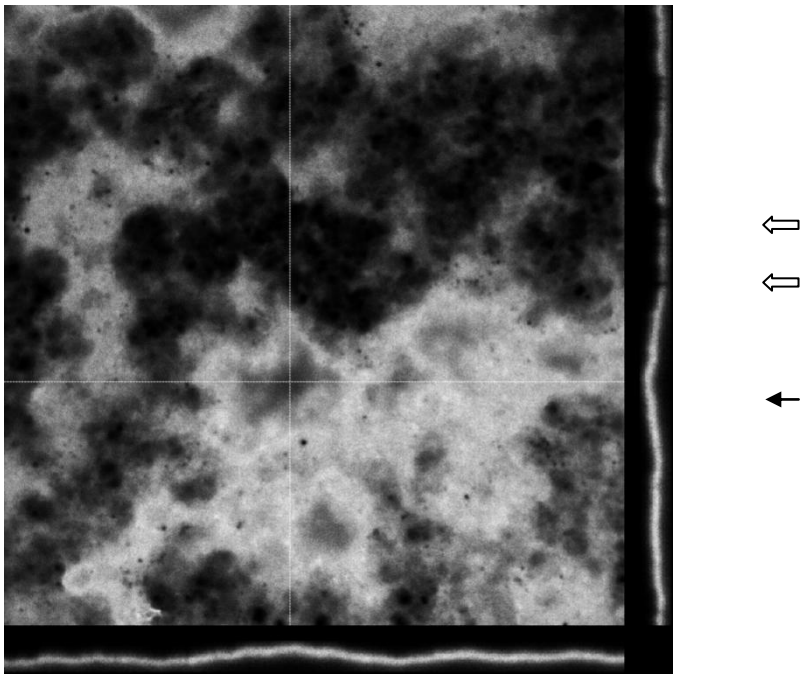


Figure 5.3 Representative orthogonal view of a 10-day-old biofilm incubated under anaerobic conditions. Laser scanning confocal microscopy image of a mature Grade IV biofilm (D (c)) at × 630 total magnifications plus × 4 zoom. The orthogonal view allows the representation of the 3 D biofilm to be presented in 2 D. Micrographs represent horizontal sections. The cross hairs indicate the area of the biofilm presented in 2 D along the borders of the image. Depicted below and to the right of the main image are the vertical yz and xz planes through the biofilm collected at the positions indicated by the lines. In this biofilm, there are channels between the microcolonies appearing as 'gaps' in the 2 D structure and indicated by the arrow (⇦). The hollow interconnecting areas under the towers are indicated by the black arrow (←).

5.3.4 Biomass and wall adherence assays

The spectrophotometric analyses of follicular fluid biofilms demonstrated an association between the number of bacterial species isolated in both aerobic and anaerobic conditions and the mean absorbance. For follicular fluid specimens from which a single cultivable bacterial species was isolated (n = 14), the resultant biofilm had the greatest mean total biomass (0.54 g ± 0.12 g) and highest mean absorbance spectrum (2.93 nm ± 0.38 nm) (Table 5.2), compared to biofilms, which comprised of two-three cultivable species. The single *Salmonella* species biofilm had a total biomass of 0.49 g (Table 5.3), but the absorbance spectrum was zero when measured against the PBS negative control. In contrast, *Lactobacillus* spp. and *S. agalactiae*

formed biofilms of similar mean total biomasses (0.49 –0.72 g) and absorbencies (2.29 – 2.62 nm). There were no overall differences in the mean total biomass when comparing bacteria isolated following aerobic or anaerobic incubation (results not shown).

Table 5.2 Biofilm total biomass and spectrophotometric analysis (mean absorbance) of *in vitro* biofilm formation in human follicular fluid

Number of species detected in biofilm culture[1]	Mean total biomass (grams)	Mean absorbance (500 nm)
One species n = 14	0.54 g ± 0.12 g	2.93 ± 0.38
Two species n = 2	0.50 g ± 0.07 g	1.93 ± .011
Three species n = 2	0.50 g ± 0.02	1.77 ± 0.39

[1] Bacteria from six of the twenty-four follicular fluids tested failed to form biofilms *in vitro*. Microbiological culture detected CoNS, *Peptostreptococcus* spp. and *E. coli* in those six follicular fluid specimens

5.3.5 Long-term *in vitro* culture of (aliquots of) follicular fluid

All follicular fluid specimens cultured *in vitro* demonstrated increasing numbers of CFUs at each subculture until eight days post incubation, after which the number of cultivable bacteria reached a plateau and remained constant for the remaining 27 weeks. From all follicular fluid specimens only a single bacterial species in pure culture may be isolated after 5-8 days incubation. With the exception of *S. entericus*, all of the bacterial species isolated after eight days were Gram-positive. Viable Gram-positive bacteria continued to be recovered from these follicular fluids incubated *in vitro* until 28 weeks.

Table 5.3 Data for 13/14 biofilms formed by a single bacterial species

Pure culture biofilm isolates	Mean total biomass (grams)	Mean absorbance (500 nm)
L. crispatus n = 10	0.49 g ± 0.17 g	2.62 ± 0.97
L. jensenii n = 1	0.54 g ± 0.16 g	2.40 ± 0.22
S. agalactiae n = 1	0.72 g	2.29
S. entericus n = 1	0.49 g	0

5.3.6 Hormonal modulators of bacterial growth in solid media and broths

When follicular fluid was cultured on solid agar media, and incubated under appropriate atmospheric conditions, either without hormones, or supplemented with hormones, there were no differences in the number of isolated CFUs. However, differences were observed in the growth patterns of bacteria cultured in thioglycollate broth (with and without hormones) for 5/12 follicular fluid specimens tested (Table 5.4). *Lactobacillus* spp. were recovered from 7/12 (58%) thioglycollate broths. For one specimen (Table 5.4, No. 12), *L. crispatus* and *L. gasseri* were cultured from the hormone supplemented thioglycollate media, but only *L. crispatus* was recovered from the hormone-free media. However, for the remaining lactobacillus containing specimens, there was no distinguishable difference in growth of the *Lactobacillus* spp. isolated in the presence or absence of hormones. *Bifidobacterium* spp. was detected in two follicular fluid specimens, and whilst the hormones supported the growth of the *Bifidobacterium* spp., this bacterium did not

grow in the absence of hormones. In contrast, the growth of *E. coli* and *S. agalactiae* was inhibited by the supplemental hormones; however, growth was observed within the original thioglycollate broth, without the addition of exogenous steroid hormones (Table 5.4)

Table 5.4 Bacterial growth in hormone supplemented thioglycollate broth

Specimen	Species identified	CFU / mL Hormone supplemented media[2]	CFU / mL Hormone-free media[2]
1	*L. gasseri*	10^6	10^6
2	*L. gasseri*	10^6	10^4
3	*L. crispatus*	10^6	10^4
4	*Bifidobacterium* spp.	10^4	No growth
5	*L. jensenii*	10^4	10^4
6	*Bifidobacterium* spp.	10^4	No growth
7	*E. coli*	No growth	$<10^3$
8	*L. crispatus, L. gasseri*	$<10^3$	$<10^3$
9	*S. agalactiae*	No growth	$<10^3$
10	No growth[1]		
11	*L. gasseri*	$>10^6$	10^6
12	*L. crispatus, L. gasseri*	10^4	*L. crispatus* only $<10^3$

[1] Culture negative, but these specimens tested positive by 16S rRNA PCR assay, [2] Thioglycollate broths

5.4 Discussion

In this study, follicular fluids collected at the time of trans-vaginal oocyte retrieval were cultured. These fluids harboured bacteria and continued to support the growth of bacteria *in vitro*. Twelve different cultivable bacterial species were detected within follicular fluid specimens, and eight of these (*L. gasseri, L. crispatus, L. jensenii, B. longum, S. agalactiae, S. anginosus, S. entericus*, and *Propionibacterium* spp.) formed biofilms *in vitro*. These results demonstrate that bacterial species, which colonise follicular fluid, or gain access to follicular fluid at the time of oocyte retrieval, may form biofilms of different biomass and complexity. With the exception of the *Lactobacillus* spp., each of the bacteria isolated in this study was a commensal microorganism found within the LGT and/or oral cavity but capable of causing opportunistic infections of the female genital tract. *Lactobacillus* spp. and *Propionibacterium* spp. were the most prevalent cultivable bacteria detected in these follicular fluids.

Biofilm formation also has been reported for vaginal (Martin *et al.*, 2008) and gastrointestinal (Fakhry *et al.*, 2009) *Lactobacillus* spp. Follicular fluid bathes the cumulus oocyte complex during maturation and prior to release at ovulation, thus bacteria within the follicular fluid may affect oocyte quality by disrupting the cumulus complex with enzymatic action or by causing DNA damage to the oocyte. Follicular fluid is a hypocoagulable, semi-viscous fluid comprised of proteins, inorganic compounds, carbohydrates, mucopolysaccharides, lipids, gonadotropins, steroid hormones, immunoglobulins, cytokines, complement components and growth factors (Edwards, 1974, Shimada *et al.*, 2001), providing a perfect growth medium for microbial populations. Furthermore, previous biofilm studies have confirmed that

microorganisms preferentially attached to hydrophobic surfaces compared to non-polar surfaces (Donlan, 2002, Donlan and Costerton, 2002, Flemming and Wingender, 2001). Thus, the hydrophobicity of human follicular fluid may further enhance biofilm formation *in vivo*.

We demonstrated that follicular fluid supported the growth of viable bacteria for a period of at least 28 weeks. This growth occurred in the absence of an exogenous addition of nutrients, or waste removal, processes that would occur *in vivo* and presumably facilitate growth even further. Gurgan *et al*. (1993) found that filter-sterilised follicular fluid was inhibitory to the Gram-positive bacteria *S. aureus; S. agalactiae and L. monocytogenes*, these species did not survive *in vitro* for longer than four days. However, Gurgan *et al*. (1993) did demonstrate that follicular fluid supported the growth of a range of Gram-negative species and the yeast *C. albicans* for up to 15 days post-inoculation. These results are inconsistent with those of the present study, which shows survival of predominantly Gram-positive species. In this current study, even though follicular fluid aliquots were not centrifuged or filtered (and therefore retained physiological composition) they continued to support the growth and recovery of primary passage viable bacterial species.

This is the first report of continuous follicular fluid culture lasting 28 weeks and it demonstrated that follicular fluid was an excellent growth medium for microorganisms. We demonstrated that bacterial colonisers of follicular fluid *in vivo*, formed biofilms within follicular fluid *in vitro*, including Gram-positive species and Gram-negative species, in 18/24 (75%) of the follicular fluids. The presence of bacteria within biofilms was dependent upon the atmospheric incubation conditions.

The incubation conditions and resultant biofilm formed reflected the growth requirements of each individual species of bacterium. *Staphylococcus* spp., *Micrococcus* spp., *Peptostreptococcus* spp. and *E. coli* were not detected in any biofilms. It was surprising to note that *E. coli* did not form a biofilm *in vitro*. Attachment and adhesion properties of *E. coli* are significantly altered throughout the menstrual cycle in response to oestradiol and progesterone however; no specific pattern has been confirmed (Venegas *et al.*, 1995). Many of the bacteria detected within follicular fluid have been shown to form biofilms *in vitro* when cultured on vaginal epithelial cells (Patterson *et al.*, 2009), *in vivo* on intrauterine devices (Pruthi *et al.*, 2003) and in amniotic fluid sludge aspirated from women with intrauterine infection (Romero *et al.*, 2008). Despite the high bacterial load recovered from follicular fluid, this fluid did not have a turbid appearance, which may suggest that bacteria within the follicle were present predominantly in biofilms rather than as planktonic bacteria. The process of trans-vaginal oocyte retrieval may result in collection of a biofilm 'plug' of bacteria within the lumen of the needle, which is subsequently then aspirated into the follicular fluid.

In keeping with the findings from the sub-cultures of follicular fluid, the majority of mature biofilms in the present study also revealed only a single cultivable species, even when two different species of lactobacillus were isolated from the follicular fluid prior to *in vitro* incubation. This may be due to competition between the lactobacilli themselves, or as a result of alterations in adhesion. It has been reported that some bacterial species can co-colonise the surface of urogenital tract cells already colonised by lactobacilli; but after lactobacilli are established, they can cause foreign bacterial cells to detach from the epithelium, which may be a defence

mechanism (Spurbeck and Arvidson, 2008, Vielfort *et al.*, 2008). In polymicrobial biofilms, early colonising species often promote the establishment of other species (Jakubovics, 2010, Jakubovics and Kolenbrander, 2010); however, whilst the initial interactions may be synergistic, once the biofilm is established, competition between species can result in the dominance of a single species (Periasamy and Kolenbrander, 2009). Furthermore, some species act as bridges between normally incompatible species, allowing them to participate in the biofilm structure (Kolenbrander *et al.*, 2006). Some microorganisms are able to scavenge O_2 creating a more favourable environment for the establishment of aerobic species in an anaerobic environment (Diaz *et al.*, 2002). This is particularly relevant to follicular fluid biofilms where the O_2 levels are low, but facultative aerobes survive. Quorum sensing, the process by which microorganisms communicate within a biofilm, establishes the overall population size of each microbial species and causes microorganisms to adjust gene expression (Monds and O'Toole, 2009). In microbial biofilms, the concentration of cell signalling molecules roughly correlates to population size for each species (Keller and Ramos, 2008). Quorum sensing by individual species occurs independently of metabolism and/or in response to metabolites secreted by individual cells for basic cellular processes (Jakubovics, 2010, Jakubovics and Kolenbrander, 2010). In addition to quorum sensing, microbial growth within the biofilm is also modulated by the metabolic cues (Kreth *et al.*, 2009). Species production of lactic acid, succinic acid and isobutyric acid reportedly enhances both the synergistic and competitive interactions between different microbial species. Acid production results in a pH shift often causing a reduction in the microbial diversity because many species are acid intolerant (Li *et al.*, 2005). The predominance of monomicrobial

biofilms (14/18) in this current study may therefore be due to the lactic acid and H_2O_2 production by *Lactobacillus* spp.

Studies of biofilms have reported that bacteria can exist in a viable, but non-cultivable state (Fux *et al.*, 2005 a). It is, therefore, possible that cultures of 10-day biofilms detected only those species that were actively replicating, while molecular techniques targeting 16S rRNA would be able to detect and quantify all species (both viable and non-viable) present within the biofilm. In this study, we performed technical replicates for each of the follicular fluid biofilm assays; however, biological replicates were not performed due to alterations that may occur in microbial count as a result of freezing and thawing of the follicular fluids in between assays. We suggest that in future studies, prior to freezing that follicular fluid is aliquotted to allow biological replicates to be performed later. To gain further knowledge of an individual species contribution to a biofilm, a potential approach is to culture and test using 16S rRNA PCR assay at various time points; so that growth curves may be established for, each individual species and culture results compared to PCR assay results. A positive 16S rRNA PCR result was obtained from one of the culture negative follicular fluid specimens investigated in this study (Specimen No. 10, Table 5.4). The 16S rRNA sequence identified an uncultured bacterium. Interestingly, non-cultivable bacteria have also been detected within uterine tube washings (data not shown). This finding confirms the presence of non-cultivable bacteria within follicular fluid and the female UGT.

An ovarian follicle has all of the requirements necessary to support the development of a biofilm. Bacterial cells that would contribute to the formation of the biofilm

have been detected. On some biofilm slides there was evidence of polysaccharide accumulation (in the absence of bacterial cells), which shows that there are components of follicular fluid (substrate) that may form a conditioning film to allow microbial attachment to the inside wall of the follicle (substratum). Furthermore, the *in vitro* development of various grades of biofilms in human follicular fluid (Figure 5.1) was demonstrated. Variation was observed in: (i) the architecture of mature biofilms (ranging from flat homogenous layers of cells through to cell clusters, microcolonies and towers with channelling and large amounts of amorphous extracellular material between the more complex heterogenous structures) (Figure 5.1 B-D (b)); (ii) the biomass, that decreased when the biofilm was comprised of more than one species (Table 5.2); and (iii) the absorption spectra, that were the highest in monomicrobial biofilms (Table 5.2 and 5.3). Mature biofilms are characterised by the development of towers and fluid–filled channels (Fey, 2010, Fey and Olson, 2010). Mature biofilms reportedly exhibit a variety of phenotypes as a result of changes in their three-dimensional structure, occurring in response to changes in cell density, osmolarity, temperature, pH and nutrient supply (Fux *et al.*, 2005a). Even follicular fluids containing the same bacterial species demonstrated different grades of biofilms (different structures and biomass). On six biofilm slides, no bacteria were observed, only a conditioning film was present. This may be due to the removal of any bacterial cells (by the PBS rinse prior to staining) present in the early stage of biofilm formation prior to permanent attachment to the conditioning film. Alternatively, the follicle may have been colonised with non-biofilm forming bacterial species. Microorganisms also produce many virulence factors, which may have synergistic or antagonistic effects on each other, the anatomical niche and in this site on the oocytes. Further studies examining the effect of these biofilm

bacterial species on each other will be beneficial to our understanding of the impact of single species and polymicrobial biofilms on reproductive health outcomes.

During an IVF treatment cycle, exogenous hormones are administered to trigger the simultaneous maturation of multiple ovarian follicles and results in elevated levels of oestradiol and progesterone in follicular fluid. Significant levels of the steroid hormones, oestradiol (303.8 - 535 ng / mL) and progesterone (735.1 - 893.2 ng / mL), have been detected in follicular fluids collected from women undergoing trans-vaginal oocyte retrieval for IVF (Loret de Mola *et al*., 1999, Kushnir *et al*., 2009). The ovarian granulosa cells are the dominant source of these steroid hormones. Granulosa cells may be aspirated with the follicular fluid and the oocyte at the time of trans-vaginal oocyte retrieval, however once removed from the ovary, the granulosa cells are no longer under pituitary control and so rapidly proceed to apoptosis (Quirk *et al*., 2011, Makrigiannakis *et al*., 2000). Whilst the initial concentrations of steroid hormones present within follicular fluid may affect *in vitro* microbial growth, the apoptotic granulosa cells would not continue to produce the significant levels of steroid hormones that would normally be produced *in vivo* (Hill and Osteen, 1989) and thus over time in culture, the impact of steroid hormone levels on microbial growth would decline.

It was demonstrated that *in vitro*, elevated levels of oestradiol and progesterone in thioglycollate broth media supported the growth of the high titres of the *Lactobacillus* spp., which were originally isolated from follicular fluid. This is consistent with *Lactobacillus* spp. growth in the LGT. At puberty, as oestrogen levels increase, the vaginal pH drops and *Lactobacillus* spp. dominate the vaginal microflora (Brabin *et al.*, 2005 b). After menopause, the vaginal microflora reverts to

the pre-menarchal state and the *Lactobacillus* spp. decline (Brabin *et al.*, 2005 a). However, if post-menopausal women receive oestrogen replacement therapy, the vaginal pH decreases and the concentration of *Lactobacillus* spp. increases (Raz, 1993). The effect of endogenous steroid hormones on lactobacilli has previously been monitored in IVF patients. Jakobsson and Forsum (2008) reported that during IVF treatment, with increasing oestrogen levels, three major vaginal lactobacilli *L. crispatus, L. gasseri* or *L. jensenii* were predominant. These reported results are consistent with our findings, as the only lactobacilli isolated from follicular fluid (collected prior to ovulation when oestradiol levels were highest) were these same species: *L. crispatus*, *L. gasseri* and *L. jensenii*. *Bifidobacterium* spp. was also isolated from the hormone-supplemented media. This species can metabolise carbohydrates from glycogen degradation in response to elevated oestradiol levels, in the same manner as *Lactobacillus* spp. (Mikami *et al*., 2009). Studies have demonstrated that the hormone concentration within maturing follicles far exceeds that found in the systemic circulation. This is driven by the hyaluronan composed cumulus-oocyte complex (Shimada *et al.*, 2001). In women undergoing ovarian hyperstimulation, follicular fluid oestradiol levels are approximately eight times higher and progesterone levels three times higher, than those for normal cycling women (Kushnir *et al.*, 2009). In this study, hyaluronidase-producing species (*Staphylococcus*, *Streptococcus* and *E. coli*) were isolated from follicular fluid; the enzymatic activity of these colonisers may lead to poor quality cumulus cells, decreased steroid hormone synthesis and the predominance of these more pathogenic bacteria within the follicle. Thus, it is indicated that high concentrations of steroid hormones within ovarian follicular fluid can influence bacterial growth.

The growth of high titre lactobacilli during IVF may be a protective mechanism within the ovary in minimising UGT infection. Reports of ovarian infection are rare, but often tubo-ovarian abscesses are reported as sequelae in pelvic inflammatory disease (Sorbye *et al.,* 2005), or secondary to respiratory tract infection with haematogenous seeding (Goulet *et al.*, 1995 b). High levels of oestradiol in hyperstimulated women should promote a 'normal' genital tract microbial flora, which has been shown to be beneficial, improving IVF outcomes. However, the success rates for IVF treatment cycles are still lower than those reported in natural conception.

The findings in this chapter have shown that (1) bacteria in follicular fluid can form biofilms *in vitro*; (2) the ovarian steroid hormones (oestradiol and progesterone) are capable of modulating *in vitro* growth of some microbial species and (3) follicular fluid supports the long-term growth of microorganisms. Further characterisation of the microorganisms detected in follicular fluid and their metabolites will increase our understanding of the effects of colonised follicular fluid on oocyte quality, on IVF fertilisation and pregnancy rates, and on early pregnancy events. Knowledge of the microorganisms present within this anatomical niche may lead to improved IVF outcomes by using targeted therapy.

CHAPTER SIX

MICROBIAL COLONISATION OF FOLLICULAR FLUID: ALTERATIONS IN CYTOKINE EXPRESSION AND ADVERSE OUTCOMES OF ASSISTED REPRODUCTIVE TECHNOLOGY

6.1 Introduction

Cytokines, chemokines and growth factors are produced by cells following activation of the innate or adaptive immune response (Husband *et al.*, 1999). Previous studies have shown that cytokines are essential for ovarian function (Richards *et al.*, 2002), modulating the secretion of ovarian steroid hormones (Richards *et al.*, 2002), and the development and regression of the corpus luteum function in humans (Chen and Peng, 2000, Sunderkotter *et al.*, 1994). Cytokines also have an essential function in embryonic development and implantation, promoting cellular differentiation, vascularisation and finally trophoblast invasion of the endometrium (Vujisic *et al.*, 2006). In recent years, a number of studies have investigated the presence of cytokines in follicular fluid and correlated these to assisted reproductive technology (ART) outcomes. These studies demonstrated that cytokines IL -1, IL-1 α, IL-1 β, IL-12/23 (p40), and vascular endothelial growth factor (VEGF) were associated with successful outcomes including increased fertilisation rates, successful embryo transfer and clinical pregnancy (Karagouni *et al.*, 1998, Vujisic *et al.*, 2006), whilst IL-12, VEGF and IL-15 were associated with poor fertilisation and failed conception (Asimakopoulos *et al.*, 2006, Gazvani *et al.*, 2000, Vujisic *et al.*, 2006). Cytokine profiles within follicular fluid also varied depending on the reproductive pathology or the cause of infertility (Amato *et al.*, 2003, Fasciani *et al.*, 2000, Hill *et al.*, 1990). To our knowledge no previous study has tested follicular fluid for microorganisms and correlated the presence of microorganisms with the cytokine profile within follicular fluid and ultimate outcomes of ART cycles.

Spandorfer *et al.* (2001) investigated abnormal vaginal flora and vaginal pro-inflammatory cytokines in women with idiopathic infertility undergoing ART cycles.

A correlation between bacterial vaginosis, elevated IL-1 β and IL-8 and idiopathic infertility was demonstrated. However, they found no correlation between the presence of bacterial vaginosis and ART pregnancy outcomes. This finding is in contrast to previous reports, which have found an association between bacterial vaginosis and second trimester miscarriage (Oakeshott *et al*., 2002); Spandorfer *et al*. (2001) suggested that antimicrobial prophylaxis following oocyte retrieval may explain this anomaly.

We hypothesised that microorganisms present in follicular fluid adversely affect oocyte quality and in addition, the microorganisms themselves or their products of metabolism elicit an immune response that results in adverse reproductive health outcomes. The aim of this study was to determine if cytokine profile(s) may be correlated with (or predict): the presence of bacteria within follicular fluid and if distinct cytokine profiles were associated with the aetiology of infertility or altered ART outcomes including oocyte retrieval rates, ART fertilisation rates and/or ART embryo transfer rates. In this current study, paired vaginal secretions and follicular fluid specimens, collected at the time of trans-vaginal oocyte retrieval were tested to detect and identify microorganisms present at these anatomical sites. Aliquots of these clinical specimens then were tested for the presence of microorganisms and 18 cytokines to compare the cytokines present in the lower and upper genital tract specimens collected from each woman and those associated with adverse ART outcomes.

6.2 Materials and Methods

6.2.1 Experimental design

In this study, a sub-cohort of 71 women from the main 263 cohort was analysed. This cohort of 71 women was selected based on the classification of their follicular fluid as: 'colonised' if microorganisms were detected within the follicular fluid but not in the vagina (at the time of oocyte retrieval); or 'contaminated' if microorganisms present in the vagina were also detected within the follicular fluid. Clinical specimens were also selected based on the couples' aetiology of infertility (Table 6.1). For each of these sub-cohorts (1) the cytokines present in the follicular fluid were compared to the presence of 'colonising' or 'contaminating' microorganisms and (2) the cytokine response was compared to the cause of infertility.

6.2.2 Specimen collection

Specimen collection was performed as previously described in Section 3.1.

6.2.3 Follicular fluid culture and colony identification

Microbial isolation and identification was performed as described in Sections 3.2 – 3.6.

6.2.4 Criteria for the selection of specimens for cytokine screening

Follicular fluids were classified as 'colonised' if microorganisms were detected within the follicular fluid but not in the vagina (at the time of oocyte retrieval); or 'contaminated' if microorganisms present in the vagina were also detected within the follicular fluid. Clinical specimens were selected and tested from 37 women with colonised follicular fluid and from 34 women with contaminated follicular fluid.

These women had a history of infertility due to endometriosis (n = 16), polycystic ovary syndrome (n = 14), genital tract infection (n = 9) or idiopathic infertility (n = 14). Specimens from 'fertile' women, from couples with male factor infertility, were selected as the control group (n = 18).

6.2.5 Cytokine testing of follicular fluid and vaginal secretions

Follicular fluid cytokine levels were measured by flow fluorimetry in 96 well microtitre plates using an automated dual laser flow cytometer (Bio-Plex Cytokine Assay System, Bio-Rad, NSW). The multiplex bead working solution was prepared for the two sets of cytokines (Bio-Rad) to be assayed: Human Group II cytokine 6-plex panel: IL-1 α, IL-12 (p40), IL-18, leukaemia inhibitory factor (LIF), macrophage colony stimulating factor (MCSF), tumour necrosis factor (TNF) β; and Human Group I cytokine 12-plex panel: IL-1 β, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-17, granulocyte colony stimulating factor (GCSF), granulocyte macrophage colony stimulating factor (GMCSF), interferon gamma (IFN γ), TNF α, and VEGF.

The follicular fluids, previously aliquotted into the cryovials containing 20 μL protease inhibitor cocktail (Sigma), were thawed on ice prior to testing. Follicular fluid samples were prepared for testing by centrifuging at 2000 × g for 20 minutes at 4º C to remove any cell debris. Blood stained follicular fluid specimens were not included in this study. The vaginal swabs were thawed on ice and rotated 20 times in 500 μL sterile phosphate buffered saline (PBS) containing 10 μL protease inhibitor cocktail. All swab preparations were performed by a single operator. Swab suspensions were then centrifuged at 2000 × g for 20 minutes at 4º C immediately prior to assay.

The standards were prepared and cytokine assays were performed as per the manufacturer's instructions. Briefly, the wells in the microtitre plate were pre-wet with 100 µL assay buffer, which was removed by vacuum filtration. Then 50 µL of bead working solution was added to each plate. Excess bead working solution was removed by vacuum filtration and the plates were washed twice in 100 µL of wash buffer. Both standards and samples were added to the plate and incubated at room temperature agitating at 300 rpm for 30 minutes. Standards and samples were assayed in duplicate. The limit of detection for this assay was determined by the manufacturer to be 2-32000 pg / mL (Bio-Rad). The plates were washed and 25 µL of detection antibody was added to each well, incubated and agitated at 300 rpm for 30 minutes. Excess antibody was removed by vacuum filtration and 50 µL of streptavidin-PE was added to each well and incubated at room temperature and agitated at 300 rpm for 10 minutes. After a final wash, the beads were resuspended in 125 µL of assay buffer and read on the (Bio-Plex 200, Bio-Rad) plate reader and analysed with software version 4.

6.2.6 Statistical analysis

Statistical analyses were performed using SPSS version 17 for Windows XP. Multinomial logistic regression was used to model the relationship between categorical outcomes and explanatory variables because all variables were not dichotomous. The continuous variables were the variables with a numerical value (the number of oocytes collected, embryos collected, those frozen, and transferred). The explanatory variables were used to explain changes in the dependent variable. For this study, the aetiology of infertility was consistently used as an explanatory variable. Microbial colonisation and contamination of human follicular fluid were

also investigated as explanatory variables for statistical analyses. The categorical variables were those variables whose values functioned as labels (embryo transfer yes/no, pregnancy yes/no). The dependent variables that were considered were colonisation (yes/no) and contamination (yes/no). Discriminant analyses were used to identify cytokines associated with: (1) the aetiology of infertility; (2) either follicular fluid 'colonised' prior to oocyte retrieval or 'contaminated' during the retrieval process; and (3) the ART outcomes for the treatment cycle. Classification and regression trees (CART) were used to identify interacting relationships between explanatory variables and either continuous or categorical response variables. In the CART analyses a minimum node size was used to determine the size of the tree. In the regression analyses, a p-value of $p < 0.05$ was considered statistically significant. All variables were entered into the model. No variables were transformed. In the discriminant analyses, Wilk's lambda was used to assess overall significance of the discriminant functions. Wilk's lambda is used for multivariate hypothesis testing and is an extension of the familiar F-test used in univariate hypothesis testing. A Wilk's lambda value close to zero indicates that group means differ. The authors note that the statistical analyses are based on a small sample size and therefore, these analyses provide indicative, not definitive results.

6.3 Results

6.3.1 Patient Demographics

Bacteria were detected in all 71 follicular fluids and vaginal secretions: and colonised and contaminated follicular fluids for each classification of infertility that was tested for this study (See Appendix 5).

The mean age of women in this study was 37 years ± 4 years. There was a statistical difference in the mean age of fertile women (35 ± 4 years) and infertile women (38 ± 4 years, $p = 0.007$). However, the age of women in this study was not associated with any adverse ART outcomes ($p > 0.05$). For fertile and infertile women there was no difference in number of prior ART treatment cycles. Fertile women participated in an average of one (range 0-3 ± 1 cycle) previous trans-vaginal oocyte retrieval procedure compared to infertile women who had participated in one (range 0-5 ± 1 cycle, $p > 0.05$). Furthermore, there was no difference in the number of oocytes collected at the time of retrieval for fertile (10 ± 5) or infertile women (11 ± 6, $p > 0.05$).

6.3.2 Culture and colony identification

The culture results for this smaller sub-cohort were consistent with those reported for the entire cohort reported in Chapter Four. A summary of the culture results for this smaller cohort is reported in Appendix 5.

6.3.3 Cytokines

The logistic regression analyses (Table 6.1) demonstrated that colonising bacteria in follicular fluid was associated with an aetiology of endometriosis ($p = 0.05$-0.1). Decreased embryo transfer rates were also observed in women with a diagnosis of polycystic ovary syndrome ($p = 0.05$-0.1). The regression model found that no follicular fluid or vaginal secretion cytokine profiles may differentiate between infertile women with a diagnosis of endometriosis, polycystic ovary syndrome or a history of upper genital tract infection when normalised against the control group of 'fertile' women (who had partners with male factor infertility). The cytokines in follicular fluid of women with endometriosis (IL-6, IL-8, IL-10 and/or GCSF),

polycystic ovary syndrome (IL-8), previous genital tract infection (IL-8) and idiopathic (IL-6, IL-10, IL-18) aetiologies of infertility were the cytokines present when normalised against the follicular fluid from 'fertile' women for all aetiologies (Table 6.1). Women with idiopathic infertility may be differentiated from all other groups by the presence of elevated levels of IL-18 in follicular fluid ($p = 0.05$-0.1). It was also determined that the presence of IL-12p40 in combination with IFN γ in follicular fluid were unique markers of successful embryo transfer ($p = 0.05$-0.1). Successful fertilisation was associated with the presence of IL-1 α ($p = 0.05$-0.1) and VEGF ($p = 0.05$-0.01) in vaginal secretions. Seventeen of the 18 cytokines tested were detected within follicular fluid, and only IL-17 was not detected (Table 6.2). Testing of vaginal secretions demonstrated the presence of 13 cytokines, but LIF, MCSF, GMCSF, IFN γ and TNF α were not detected (Table 6.2). There were site-specific differences in the levels of cytokines detected in the follicular fluids compared to the vaginal secretions, particularly for the pro-inflammatory cytokines: IL-1 β (vaginal secretions, Figure 6.2), IL-8 (vaginal secretions, Figure 6.3) and IFN γ (follicular fluid, Figure 6.2).

Table 6.1 Regression analyses of the cytokines detected in follicular fluid and vaginal secretions correlated with colonising or contaminating microorganisms, the aetiology of infertility and ART outcomes

	ART outcomes		**Microorganisms**	**Cytokines**									
Aetiology of infertility	Decreased embryo transfer rate	Decreased fertilisation rate	Colonisers	IL-1 α	IL-12 p40	IL-18	IL-1 β	IL-6	IL-8	IL-10	GCSF	IFN γ	VEGF
Endometriosis n = 16	NS	NS	†	NS	NS	NS	(†)	†	† (†)	† (†)	†	NS	NS
PCOS n = 14	†	NS	NS	NS	NS	NS	(†)	NS	**†(*)**		(†)	NS	NS
UGT infection n = 9	NS	NS	NS	NS	NS	NS	(†)	NS	†	(†)	(†)	NS	NS
Male factor n = 18	NS	NS	NS	NS	NS	NS	NS	NS	NS		NS	NS	NS
Idiopathic n = 14	NS	NS	NS	NS	NS	†	(*)	* (†)	(†)	*(*)	NS	NS	NS
ART outcomes	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Embryo transfer[2]	NS	NS	NS	NS	†	NS	(†)	†	NS	NS	†	†	NS
Fertilisation rate[3]	NS	NS	NS	(†)	NS	†	(†)	NS	NS	NS	NS	NS	**(**)**
Microorganisms	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Colonisers	NS	NS	NS	NS	NS	NS	NS	NS	NS	† (†)	NS	NS	†

[1] Cytokines expressed within follicular fluid in **bold,** cytokines expressed within vaginal secretions in brackets (), [2] Successful embryo transfer, [3] Successful fertilisation rate, PCOS = polycystic ovary syndrome, † p = 0.05 - 0.1; * p < 0.05; ** p < 0.01; NS = not significant

Table 6.2 Mean cytokine concentrations detected in follicular fluid and vaginal secretions from women undergoing IVF

Cytokines	Follicular fluid cytokines (pg / mL)	Vaginal secretion cytokines (pg / mL)
IL-1 α	0.16 ± 0.69[1]	0.75 ± 1.43
IL-12 (p40)	9.08 ± 26.31	4.92 ± 28.60
IL-18	1.48 ± 3.12	1.05 ± 5.86
LIF	0.01 ± 0.05	0
MCSF	0.44 ± 2.32	0
TNF β	0.14 ± 1.10	1.41 ± 7.53
IL-1 β	0.25 ± 0.81	7.18 ± 24.80
IL-4	0.53 ± 2.62	0.10 ± 0.87
IL-6	20.70 ± 14.58	3.51 ± 6.71
IL-8	101.68 ± 209.53	391.41 ± 1090.58
IL-10	28.82 ± 36.11	3.85 ± 9.96
IL-12 (p70)	5.06 ± 13.58	0.48 ± 3.16
IL-17	0	0.86 ± 6.97
GCSF	5.46±18.69	16.11 ± 42.59
GMCSF	0.10±0.59	0
IFN γ	880.66 ± 2308.55	0
TNF α	4.33 ± 27.52	0
VEGF	1364.93 ± 1621.35	234.04 ± 829.52

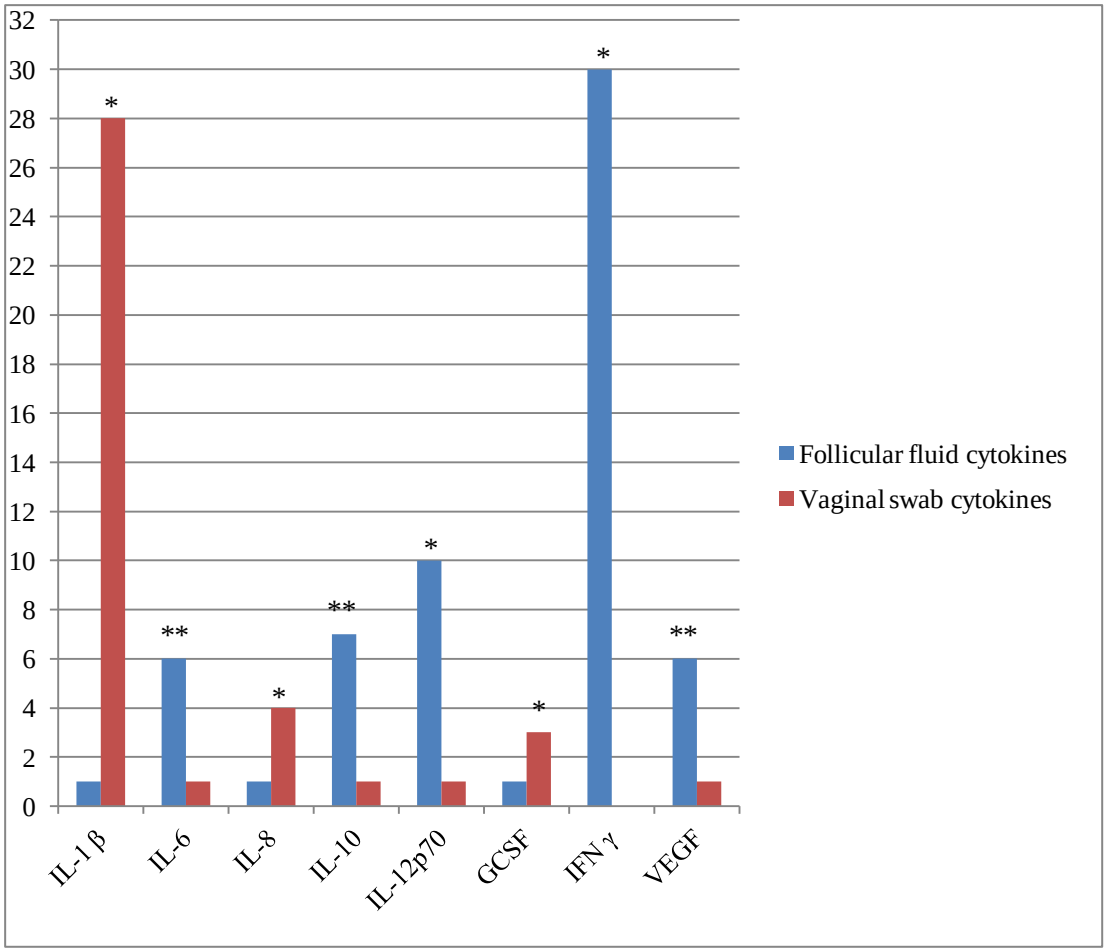


Figure 6.1 Fold-change between follicular fluid cytokines and vaginal secretion cytokines. Genital tract cytokines were differentially expressed within follicular fluid and vaginal secretions. Figure 6.1 represents the measured cytokines (in pg / mL) expressed as a fold-change for each genital tract site (follicular fluid and vaginal secretions). Expression of IL-1 ß, IL-8 and GCSF occurred at higher levels in the vaginal secretions. Elevated levels of IL-6, IL-10, IL-12p70, IFN γ and VEGF were detected in follicular fluids.* $p < 0.05$, ** $p < 0.001$

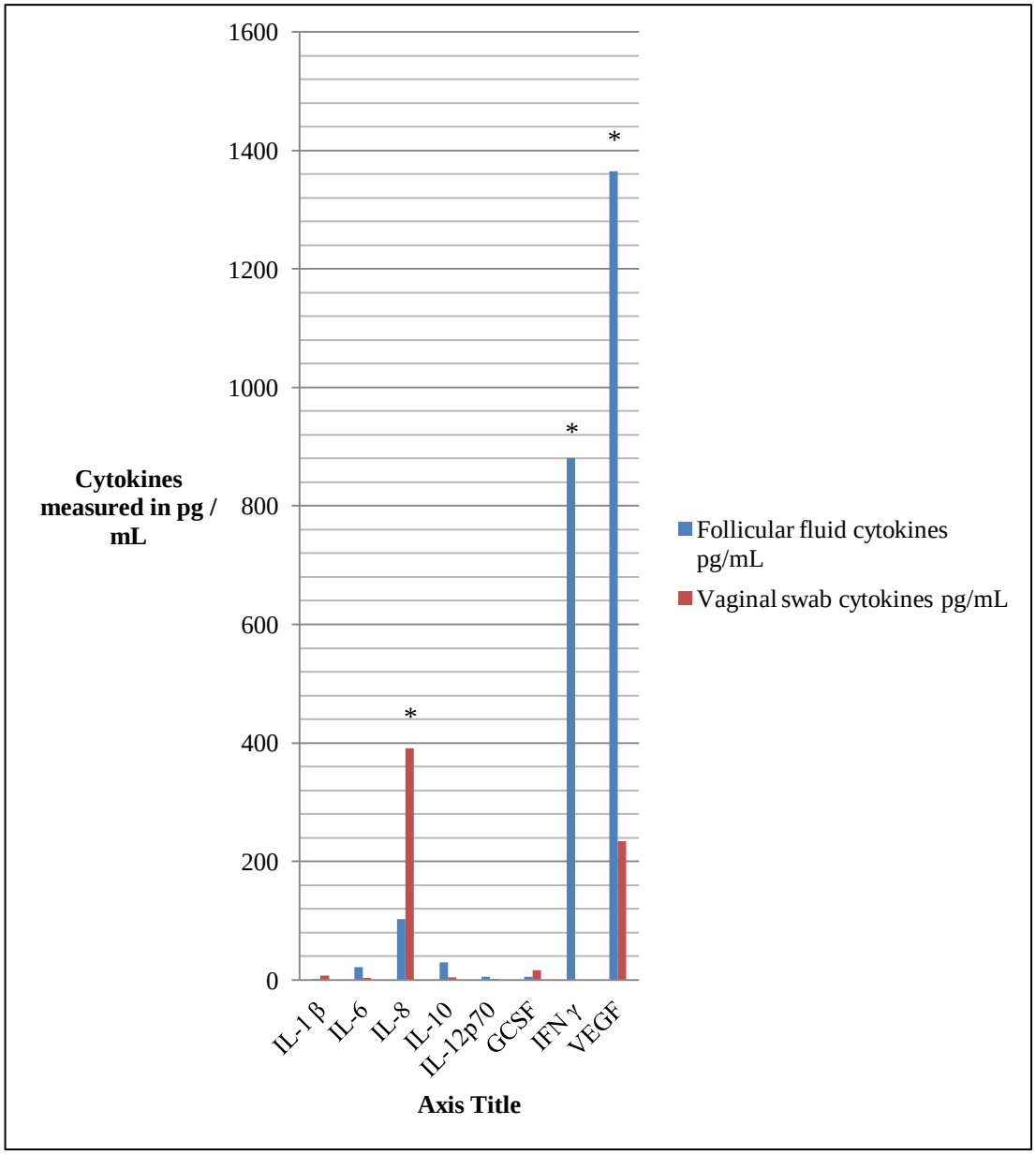


Figure 6.2 Concentration of follicular fluid and vaginal secretion cytokines. Genital tract cytokines were differentially expressed within follicular fluid and vaginal secretions, giving significant differences between each the expression at each site. The concentration of IFN γ and VEGF was much higher in follicular fluid specimens compared to vaginal secretions. The IL-8 concentration was higher in the vagina. * $p < 0.05$

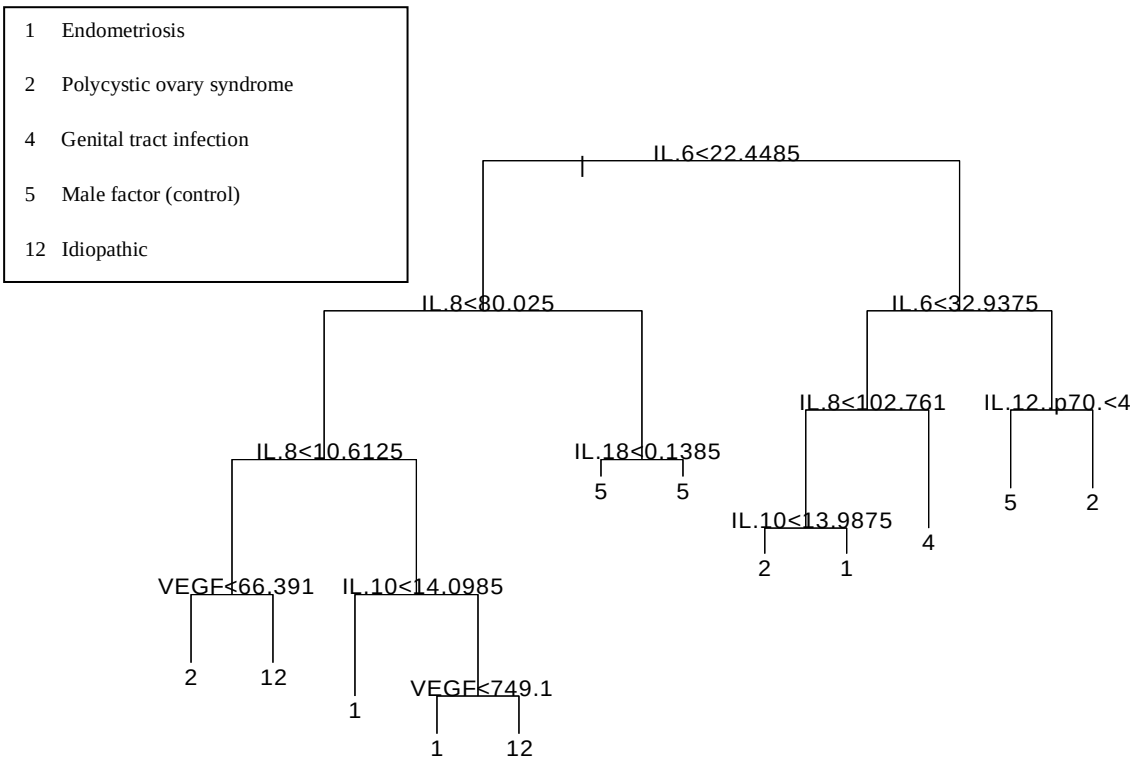


Figure 6.3 CART analysis of the cytokines associated with the different aetiologies of infertility. Classification and regression analysis (CART) analysis revealed that six follicular fluid cytokines: IL-6, IL-8, IL-12p70, IL-18 IL-10, and VEGF, may be used to predict a relationship between cytokines and the aetiology of infertility.

Classification and regression tree (CART) analysis (Fig 6.3) revealed that six follicular fluid cytokines (IL-6, IL-8, IL-12, IL-18 IL-10, and VEGF) may be used to predict a relationship between cytokines and the aetiology of infertility. This analysis was also useful for selecting cytokines (IL-1 β, VEGF, IL-10, IL-8, IL-6, IL-18,), which may be markers for embryo transfer (Fig 6.4). For example, CART analysis (Figure 6.3) revealed that the following combinations of variables: IL-6 (< 22.4485 pg / mL), IL-8 (< 10.6125 pg / mL) and VEGF (> 66.391 pg / mL) or IL-6 (< 22.4485 pg / mL), IL-8 (< 10.6125 pg / mL), IL-10 (> 14.0985 pg / mL) and VEGF (> 749.1 pg / mL) were predictive of an idiopathic aetiology (Fig 6.3 (12)) of

infertility. Successful embryo transfer was indicated by IL-1 β (< 0.191 pg / mL), VEGF (< 219.903 pg / mL) and IL-10 (< 3.06 pg / mL) or IL-1 β (< 0.191 pg / mL), VEGF (> 219.903 pg / mL but < 801.173 pg / mL) and IL-8 where IL-1 β and VEGF are the most important cytokines (indicated by the long tree branches). The relative importance of each cytokine is indicated by the length of the tree branch (Fig 6.3). There appears to be substantial interactive effects between the cytokines in differentiating both the aetiology of infertility and embryo transfer, as shown by the network of branches, which differ by combinations of variables.

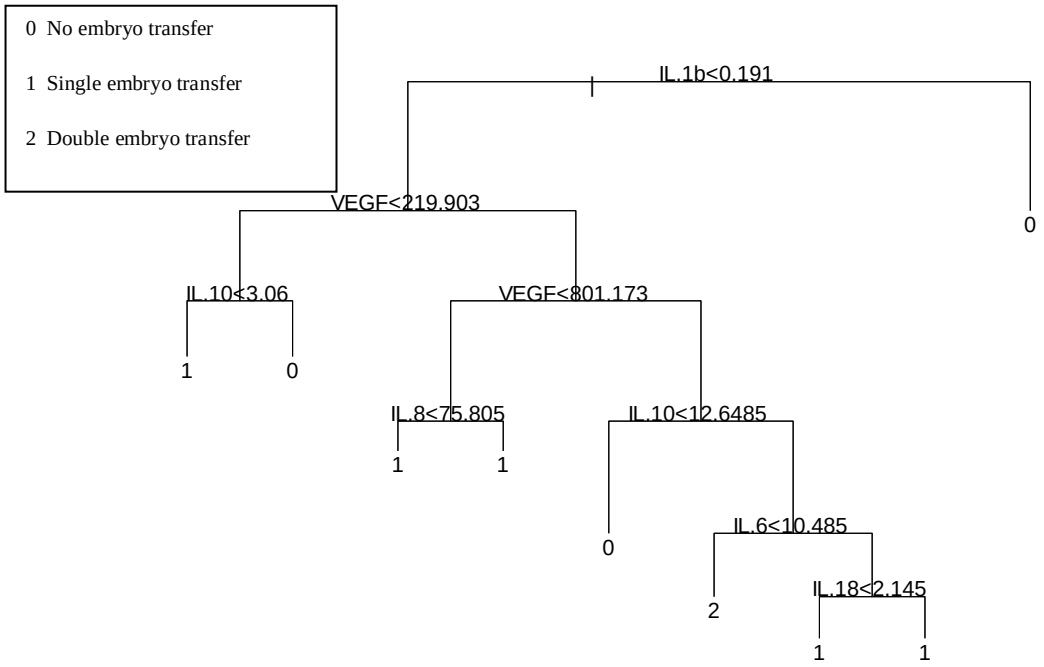


Figure 6.4 CART analysis of cytokines associated with embryo transfer. CART analysis revealed cytokines (IL-1β, VEGF, IL-10, IL-8, IL-6, IL-18), which may be markers for failed, or successful (single or double) embryo transfer.

Table 6.3 (A) Discriminant analysis of cytokines – follicular fluid

Discriminant analysis	**Aetiology of infertility**	**Wilk's Lambda**	**Fertilisation rate**	**Wilk's Lambda**	**Embryo transfer rate**	**Wilk's Lambda**
A. Follicular fluid						
Cumulative percentage (1^{st} and 2^{nd} functions)	83.7%		100%		100%	
follicular fluid cytokines (1^{st} function)	MCSF IL-4 IL-10	0.177	MCSF TNF β GMCSF IL-10 LIF	0.743	IL-8	0.469
follicular fluid cytokines (2nd function)	LIF IL-10 TNF β VEGF MCSF	0.418	N/A		N/A	0.713
follicular fluid cytokines (2nd function)	LIF IL-10 TNF β VEGF MCSF	0.418	N/A		N/A	0.713

[1] Mean ± SD

Discriminant analyses (Table 6.3) were performed to compare the aetiology of infertility, fertilisation rate and embryo transfer rates using both follicular fluid cytokine levels and vaginal secretion cytokine levels. No results presented reached significance, however; the first discriminatory function for follicular fluid indicates that > 80% of variation in aetiology is coming from between group variation. Discriminant analysis of follicular fluid found two major discriminatory functions (groups of cytokines), which classified 83.7% of the groups for infertility aetiology (Table 6.3 - A). These were not significant by Wilk's Lambda but were highly weighted (by the Eigen value) suggesting that there may be physiological interactions occurring between: MCSF, IL-4, IL-10 (first function aetiology) and LIF, IL-10, TNF β, VEGF and M-CSF (second function aetiology, page 182). Only a single function was required for discriminating between 100% of successful and unsuccessful outcomes for the fertilisation rate and embryo transfer rate groups: M-CSF, TNF β, GMCSF, IL-10, LIF (first function fertilisation rate); and IL-8 (first function embryo transfer, page 182). Whilst these were again not significant by Wilk's Lambda they were also highly weighted. A similar trend was observed with the vaginal secretion discriminant analysis. The analysis found two major discriminatory functions, which classified 80% of aetiology groups: IL-1 β and IL-8 (first function) and IL-1 β, IL-8 and GCSF (second function) (Table 6.3 – B, page 184). Fertilisation rate and embryo transfer rate groups again required only a single function to discriminate between 100% of the group: IL-1 β (first function fertilisation rate) and MCSF, TNF β and IL-4 (first function embryo transfer). Wilk's Lambda was not significant for any discriminant functions of vaginal secretion cytokines.

Table 6.3 (B) Discriminant analysis of cytokines – vaginal secretions

Discriminant analysis	**Aetiology of infertility**	**Wilk's Lambda**	**Fertilisation rate**	**Wilk's Lambda**	**Embryo transfer rate**	**Wilk's Lambda**
B. Vaginal secretion						
Cumulative percentage (1st and 2nd functions)	80%		100%		100%	
VS cytokines (1st function)	IL-1 β IL-8	0.399	IL-1 β	0.718	MCSF TNF β IL-4	0.663
VS cytokines (2nd function)	IL-1 β IL-8 GCSF	0.643	N/A		N/A	

[1] Mean ± SD

The functions at group centroid indicate that follicular fluid and vaginal secretion cytokine profiles for fertilisation (Figures 6.5 A and B) and embryo transfer (Figures 6.6 A and B), both group in the same manner and samples of either would be suitable markers to predict these ART outcomes, albeit using different cytokine profiles. By contrast, follicular fluid and vaginal secretion cytokines group aetiologies of infertility very differently (Figures 6.7 A and B). Follicular fluid cytokines readily differentiate women with a history of upper genital tract infection from all other

groups (Fig 6.7 A). This may represent the overt inflammatory response associated with long term sequelae as a result of upper genital tract infection. Vaginal fluid cytokines distinguish women from male factor (control group) couples from women with all other causes of infertility (Fig 6.7 B). This may be a useful non-invasive early screening test for women from partnerships where the males are infertile.

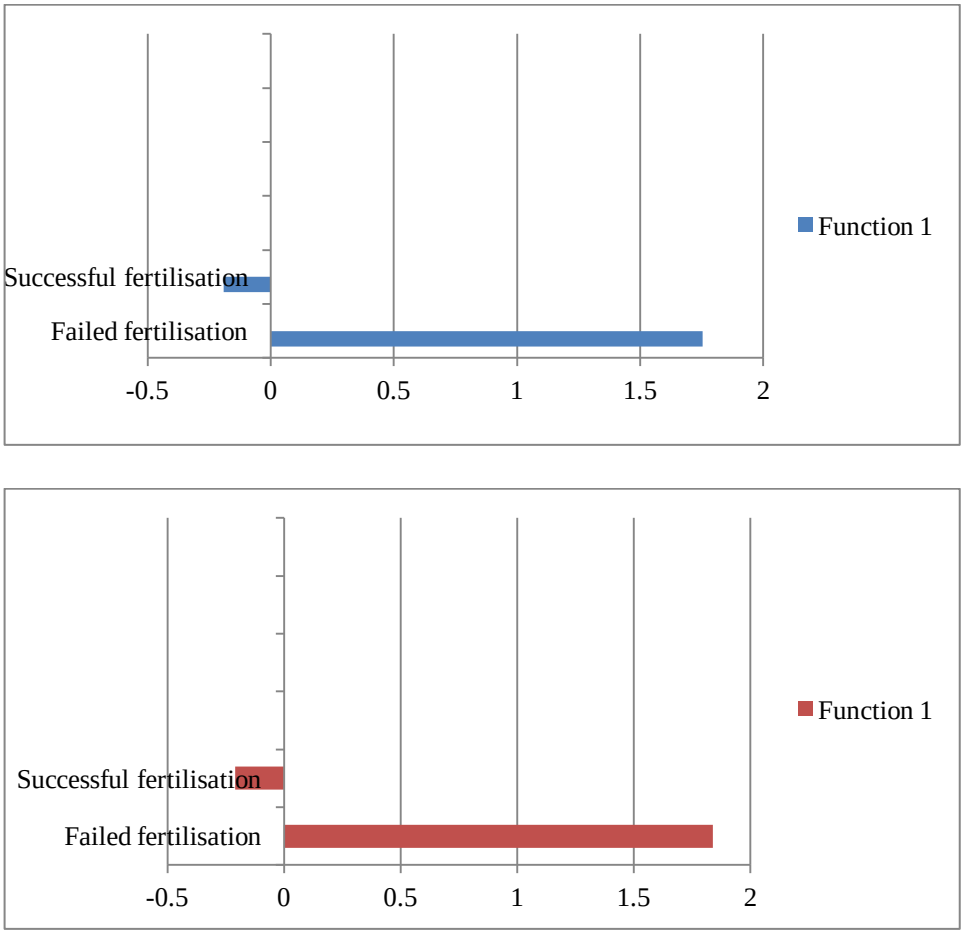


Figure 6.5 Functions at group centroid for A- follicular fluid cytokines and B - vaginal secretion cytokines and fertilisation. The functions at group centroid provide a graphical representation of the ability of follicular fluid (blue) or vaginal secretion (red) cytokines to predict ART outcomes. For fertilisation outcome, the model indicates that there are cytokines expressed at each anatomical site that are associated the fertilisation outcome. The discriminant analysis indicates the cytokines at each site.

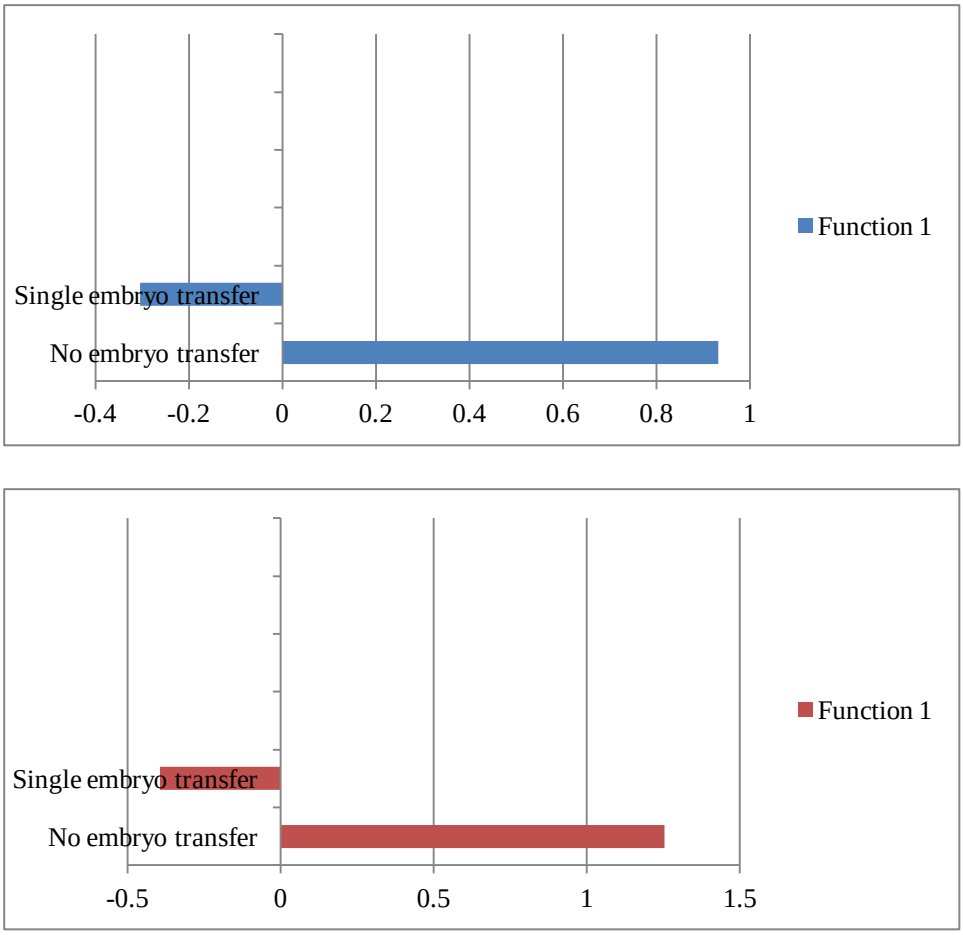


Figure 6.6 Functions at group centroid for A- follicular fluid cytokines and B - vaginal secretion cytokines and embryo transfer. The functions at group centroid provide a graphical representation of the ability of follicular fluid (blue) or vaginal secretion (red) cytokines to predict embryo transfer outcomes. For embryo transfer, the model indicates that there are cytokines expressed at each anatomical site that can successfully predict the embryo transfer outcome.

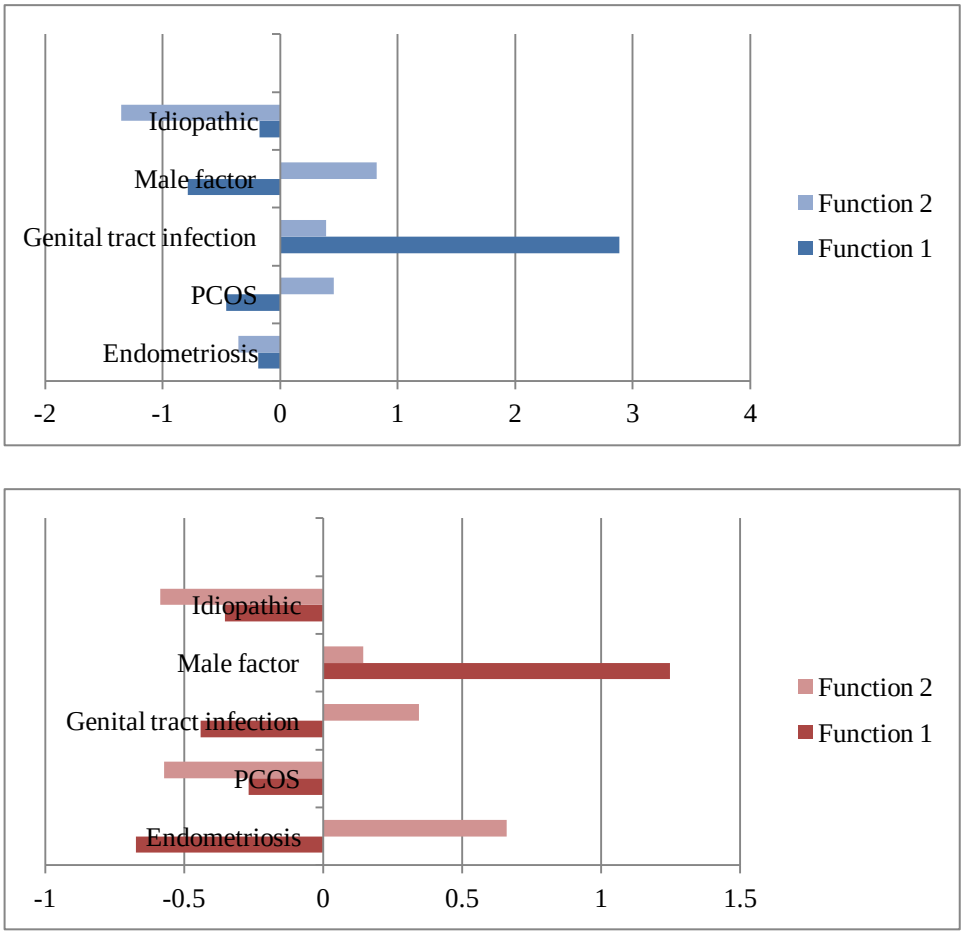


Figure 6.7 Functions at group centroid for A- follicular fluid cytokines and B - vaginal secretion cytokines and aetiology of infertility. The functions at group centroid provide a graphical representation of the ability of follicular fluid (blue) or vaginal secretion (red) cytokines to predict the aetiology of infertility. The model indicates that there are distinct differences in cytokine expression at each anatomical site for each cause of infertility.

6.4 Discussion

This study has confirmed that cytokine profiles may be useful in predicting IVF outcomes. We reported that a single cytokine (IL-18) was discriminatory for women with an idiopathic aetiology of infertility. Previous studies have reported cytokine profiles for ovarian conditions including endometriosis and polycystic ovary syndrome (Amato *et al.*, 2003, Fasciani *et al.*, 2000, Hill, 1993). In this study, we have confirmed previous findings but in addition, we have compared cytokine

profiles of women with microbial colonised and contaminated follicular fluid and different causes of infertility, with their IVF outcomes.

This study identified unique follicular fluid and vaginal secretion cytokine levels in women with idiopathic infertility (Table 6.2). In a study of women with idiopathic infertility, Spandorfer *et al.* (2001) reported elevated levels of IL-1 β and IL-8 in cervical secretions from women with bacterial vaginosis but they found no correlation between altered vaginal flora and IVF outcomes: this may be because women received prophylactic antimicrobial treatment following oocyte retrieval. We detected these same cytokines (IL-1 β and IL-8) and in addition IL-6 and IL-10, in vaginal secretions and IL-6, IL-10 and IL-18 within follicular fluid from women with idiopathic infertility. IL-18 (previously known as interferon inducing factor) was a discriminating cytokine detected only in the follicular fluid of women with idiopathic infertility in this study. This suggests that there may be a pathological cause for this infertility. Kilic *et al*. (2009) examined IL-18 as a marker of adverse IVF outcomes and not a predictor of idiopathic infertility. They reported a negative correlation between the levels of follicular fluid IL-18 and oestradiol in a subset of obese women with idiopathic infertility. More recently, excessive oestradiol levels reportedly inhibit the expression of IL-18 within the endometrium (Ashworth *et al*., 2010). IL-18 levels were found to be higher in the uterine flushings of women with idiopathic infertility when compared to women undergoing IVF for male or tubal factor infertility (Oger *et al*., 2009). Therefore, it appears that there is a similar relationship between IL-18 and oestradiol within both the ovary and the endometrium. IL-18 has also been identified in genital tract infections. Hook *et al*. (2005) demonstrated that elevated levels of both IL-12 and IL-18 acted synergistically to produce the

necessary IFN γ response required to clear genital tract infections caused by *Chlamydia trachomatis* and in addition, bacterial vaginosis in women with idiopathic infertility was also associated with significantly higher levels of cervical IFN γ. IL-18 has also previously been reported within the semen of infertile men with a history of genital tract infection. However, it was not associated with any alterations in semen andrology parameters (Matalliotakis *et al.*, 2006). Together, these reports suggest that IL-18 may be a marker of asymptomatic genital tract infection, which in infertile couples may have been classified as idiopathic infertility.

IL-18 also appears to be an integral cytokine in the establishment of a successful pregnancy. In previous studies, the ratio of IL-15 and IL-18 to tumour necrosis factor weak inducer of apoptosis was found to be a key factor in uterine receptivity at the time of embryo transfer, rather than the normalised values of each of these cytokines (Ledee *et al*., 2011, Petitbarat *et al*., 2010). Together, these observations suggest that both the follicular fluid and the endometrial IL-18 expression may play an integral role in the establishment of a successful ongoing pregnancy. Cytokine screening of vaginal secretions and follicular fluid specimens during an IVF treatment cycle may therefore offer rapid, valuable immuno-modulatory information, in addition to the standard morphological assessment of embryos. Cytokine profiles related to successful IVF outcomes may further inform the selection of good quality embryos for transfer to the uterus.

The inactive precursor of IL-18 is reportedly expressed in various cell types including macrophages. The inactive pro-IL-18 is cleaved by caspase-1 or proteinase -3 to form the biologically active mature IL-18 (Orengo *et al*., 2011). In addition to

IFN γ induction, the pro-inflammatory characteristics of IL-18 also result in enhancement of T and NK cell maturation, cytokine production and cytotoxicity. The chemotactic activity of IL-18 may be a reason for the large number of lymphoid cells within the ovary. Cells from the white cell lineage including monocytes, macrophages, lymphocytes, neutrophils and eosinophils have been described within ovarian tissues (Norman and Brannstrom, 1996). Of these, macrophages and monocytes account for 5-15% of the cell types within follicular fluid (Loukides *et al.*, 1990) and as a result many studies have investigated the role of follicular fluid cytokines on the physiology of reproduction and IVF outcomes. Macrophages are present in ovarian tissues and are activated during an immune response to pathogens. These immune effectors also play a significant role in homeostasis via cytokine production and secretion (Gordon and Rowsey, 1999). During an endometrial cycle, the secretion of inflammatory (IL-1, IL-6, TNF α) and anti-inflammatory (IL-10) cytokines and growth factors in endometrial tissues facilitate cellular proliferation and angiogenesis (IL-6, IL-8, VEGF) necessary for the cyclical changes from the proliferative (follicular) through to the secretory (luteal) phase, in preparation for implantation and ongoing pregnancy (Wu *et al.*, 2004). Similarly, macrophages control events within the ovarian follicles and have been detected within the ovary in fluctuating numbers throughout the menstrual cycle (Brannstrom *et al.*, 1994). During follicle growth, macrophage numbers within the ovary proliferate and localise to dominant (maturing) follicles (Wu *et al.*, 2004), secreting cytokines to stimulate cellular proliferation (IL-1 α, IL-1 β), follicular growth, steroid hormone production and secretion (IL-2, IL-6, LIF, TNF α) and the suppression of apoptosis (IL-1 β) (Richards *et al.*, 2002). An increase in macrophage numbers within the ovarian theca just prior to ovulation also results in the secretion of proteases and

cytokines thought to be essential for ovulation to occur (Brannstrom *et al.*, 1995, Tadros *et al.*, 2001). Furthermore, the formation and regression of the corpus luteum is modulated by macrophage-secreted cytokines, those associated with angiogenesis (Sunderkotter *et al.*, 1994) and increased production of progesterone (Chen and Peng, 2000).

Natural killer cells are also present in high numbers within follicular fluid collected at the time of trans-vaginal oocyte retrieval and are capable of modulating cytokine expression (Krizan *et al.*, 2009). An alteration in the level of immune-regulatory follicular fluid natural killer cells and natural killer T cells has been correlated with IVF outcome (Fainaru *et al.*, 2010, Krizan *et al.*, 2009). This may suggest that women with impaired natural killer cell function may have an impaired follicular fluid cytokine profile and also an impaired ability to respond to viral challenges and this may further contribute to their infertility. It should be noted, however, that the follicular fluid cytokine regulation by natural killer cells would remain unaltered in response to bacteria.

Alterations in the cytokine levels and profiles have been reported in follicular fluid and ovarian tissue for women with endometriosis (Fasciani *et al.*, 2000), polycystic ovary syndrome (Amato *et al.*, 2003), premature ovarian failure (Hill *et al.*, 1990) and ovarian cancer (Pisa *et al.*, 1992). This current study confirmed the association between four follicular fluid cytokines (IL-6, IL-8, IL-10 and GCSF) in women with endometriosis and also three vaginal fluid cytokines (IL-1 β, IL-8 and IL-10) (Table 6.1) previously reported in women with an aetiology of infertility of endometriosis (Fasciani *et al.*, 2000, Salmassi *et al.*, 2005, Van Blerkom *et al.*, 1997).

Endometriosis is an inflammatory condition involving the ectopic development of transplanted endometrial tissue (Speroff and Fritz, 2005). This tissue has potent angiogenic properties with the ability to progress and infiltrate many ectopic sites, and this is modulated by altered functions in immune response cells (Ho *et al.*, 1997). In women with endometriosis, there is also an increased expression of the cytokines IL-1 β, IL-6, IL-8 and IL-10 in peritoneal fluid (Ho *et al.*, 1996, Wu and Ho, 2003) and IL-6, IL-10, GCSF and VEGF in follicular fluid (Kilic *et al.*, 2007, Pellicer *et al.*, 1999). IL-6 (Motro *et al.*, 1990) and IL-8 (Koch *et al.*, 1992) have also been reported in association with angiogenesis when cytokine expression levels were correlated with the growth of new vasculature, endometrial cells, ectopic endometrium (Arici *et al.*, 1998) and disease severity (Gazvani *et al.*, 1998). High levels of angiogenic factors (VEGF (Fig 6.2), IL-6 and IL-8 (Fig 6.2 and Table 6.2)) reported within follicular fluid in this current study are further evidence of the angiogenic activity surrounding the ovarian follicles.

In this study, women with polycystic ovary syndrome demonstrated increased expression of follicular fluid cytokine levels of IL-8 and vaginal fluid cytokine levels of IL-8 and GCSF. Whilst these same patterns of cytokine expression have previously been reported for women with polycystic ovary syndrome (Amato *et al.*, 2003), others found no detectable differences between cytokines when comparing women with polycystic ovary syndrome to those without polycystic ovaries (Jasper and Norman, 1995, Pellicer *et al.*, 1999).

Ge and You (2008) demonstrated expression of an IL-17 receptor in oocytes, but the function of this cytokine in the ovary is not known and in this current study IL-17 was not detected within follicular fluid.

This chapter reported the presence of cytokine profiles, which were associated with adverse IVF outcomes including decreased fertilisation rates and decreased embryo transfer rates and we propose that the cytokine profiles were generated in response to the colonising or contaminating bacteria present within follicular fluid. The cytokines we detected within follicular fluid and vaginal secretion specimens, which were associated with fertilisation and embryo transfer outcomes were from both the pro-inflammatory (IL-6, IL-8, IL-12p40, LIF, IFN γ and IL-18) and anti-inflammatory (IL-4, IL-10, GCSF) cytokine groups (Tables 6.1 and 6.2). However, it is interesting to note that none of the follicular fluid specimens tested in this study appeared infected on gross examination. Similarly, women with bacterial vaginosis and idiopathic infertility most frequently have no symptoms of infection but demonstrate an altered cytokine profile in their cervical secretions (Cherpes *et al.*, 2008). However, the overall pro-inflammatory to anti-inflammatory cytokine balance in women with asymptomatic bacterial vaginosis remains unchanged and it has been proposed that the balanced immune response may be responsible for the lack of symptoms (Cherpes *et al*., 2008). Therefore, in this study we have demonstrated that infertile women without any pathological symptoms have altered follicular fluid cytokine profiles, which were associated with adverse IVF outcomes.

It is proposed that the alteration in the immune response in women with colonised follicular fluid may be a previously undescribed cause of infertility. Data presented

in this chapter suggests that (1) cytokines are expressed at different site-specific levels in the genital tract (vagina and follicular fluid), (2) genital tract cytokines may be biomarkers of the aetiology of infertility, (3) IL-18 may be a useful diagnostic for couples with idiopathic infertility, (4) a vaginal secretion cytokine profile may predict successful fertilisation and (5) a follicular fluid cytokine profile may be a useful predictor of fresh embryo transfer in an IVF cycle. The identification of a cytokine profile, which predicts adverse IVF outcomes including failed fertilisation, failed embryo transfer, failed conception or early pregnancy loss may lead to the development of targeted therapy to modulate the immune response within the upper genital tracts of these women, which may potentially improve IVF pregnancy outcomes.

CHAPTER SEVEN

GENERAL DISCUSSION

7.1 Major findings of this thesis

1. Microbiological culture methods were used to demonstrate that approximately 30% of human follicular fluid specimens are colonised by a diverse range of microorganisms. In these women, the microflora of follicular fluid and vaginal mucosa varied. Women with colonised follicular fluid had decreased fertilisation rates, embryo transfer rates and pregnancy rates when compared to women with follicular fluid contaminated by vaginal microorganisms.

2. The propensity of many species isolated from follicular fluid to form biofilms *in vitro* offers a mechanism by which such microorganisms can persist within follicular fluid despite its antimicrobial properties. Follicular fluid was able to support microbial growth of numerous species for an extended period up to 28 weeks.

3. The host's immune response, measured by cytokine expression in this study, identified site-specific cytokine profiles for the follicular fluids from the UGT and vaginal secretions from the LGT in both fertile and infertile women. A vaginal secretion profile, which may be useful for predicting successful fertilisation was identified, as was a follicular fluid profile, which may be useful for predicting successful embryo transfer. Elevated follicular fluid IL-18 and decreased IL-10 levels were found to be significantly associated with idiopathic infertility. Colonising bacteria were associated with elevated follicular fluid VEGF levels, and both were associated with infertility due to endometriosis.

Table 7.1 Major findings of the thesis

Thesis chapter	Major findings	Reference
Chapter Four	Follicular fluid is not sterile and contains a diverse range of microorganisms	Tables 4.6, 4.7 and 4.8 Figure 4.1
	Follicular fluid may be (1) colonised or (2) contaminated	Tables 4.2, 4.6 and 4.13
	Colonisation and contamination of follicular fluid are not related to the aetiology of infertility	Tables 4.2, and 4.13
	Colonised follicular fluid is associated with adverse IVF outcomes	Tables 4.5, 4.6, and 4.8
	Species diversity is higher in the vagina than in follicular fluid	Table 4.3
	Microbial load is not the most significant factor in adverse IVF outcomes	Table 4.4
	Lactobacillus spp. is not associated with adverse IVF outcomes	Table 4.8 and 4.12
	Some follicular fluid microorganisms are opportunistic pathogens associated with adverse IVF outcomes	Table 4.8 and 4.12

	Bacteria detected in follicular fluid can form biofilms *in vitro*	**Tables 5.1, 5.2 and 5.3 Figures 5.1 and 5.2**
Chapter Five	Steroid hormones modulate *in vitro* microbial growth	Table 5.4
	Follicular fluid supports the long term growth of microorganisms	pp 155
Chapter Six	Genital tract cytokines are expressed in a site-specific manner	Tables 6.2, and 6.3 Figures 6.1 and 6.2
	Colonisation of follicular fluid is related to adverse IVF outcomes	Table 6.1 and 6.3 Figure 6.7
	Follicular fluid and vaginal secretion cytokine profiles are useful in predicting the aetiology of infertility	Table 6.3
	IL-18 may be a diagnostic marker for idiopathic infertility	Tables 6.1
	A vaginal secretion cytokine profile can predict successful fertilisation	Table 6.3
	A follicular fluid cytokine profile can predict successful embryo transfer	Tables 6.1 and 6.3 Figures 6.4 and 6.6

7.2 Discussion

Follicular fluid from asymptomatic women often was not sterile and contained a diverse range of microorganisms. These microorganisms were either colonisers or contaminants from the LGT. The presence of colonising microorganisms in follicular fluid appeared to be related to adverse IVF outcomes. Whilst progress has been made in increasing IVF fertilisation rates in infertile couples, this has not translated to similar levels of successful pregnancy outcomes (Wang *et al.*, 2010). Previous studies have investigated causes of unsuccessful IVF treatments, and have identified microorganisms present in both male and in female UGTs and LGTs (as opportunistic pathogens from the normal regional flora, contaminants or STIs) (Benchaib *et al.*, 2003, Borini *et al.*, 2006, Eckert *et al.*, 2003, Egbase *et al.*, 1996, 1999, Fanchin *et al.*, 1998, Henkel *et al.*, 2004, Moore *et al.*, 2000). Our studies have confirmed previous reports that the female upper genital tract is not a sterile site (Moller *et al.*, 1985, Spence *et al.*, 1982, Viniker, 1999).

The process of trans-vaginal oocyte retrieval for IVF is a procedure by which contaminating microorganisms may be introduced into follicular fluid. However, infection following trans-vaginal oocyte retrieval is rare, and previous studies assumed that the vagina was the source of bacteria even though the same species were not always isolated from the corresponding needle flush (media flushed through the oocyte retrieval needle at the end of the collection) (Cottell *et al*., 1996). Therefore, despite the identification of bacteria in human follicular fluid over a decade ago, previous investigations failed to define 'colonising' species in this fluid (Cottell *et al*., 1996). Another study correlated the presence of follicular and peritoneal fluid microorganisms with pelvic infection following laparoscopic tubal

embryo transfer (Saltes *et al*., 1995) and the authors concluded that the microorganisms present in these fluids had no affect on pregnancy rates, nor were they associated with pelvic infection (Saltes *et al*., 1995). Consequently, previous investigations on small numbers of samples perhaps too quickly discounted follicular fluid as a source of adverse reproductive health outcomes.

This study characterised the microflora in follicular fluid specimens collected from 262 women (undergoing fully stimulated IVF cycles) at the time of trans-vaginal oocyte retrieval and found that the presence of colonising bacteria in follicular fluid was associated with adverse IVF outcomes. The microflora was diverse, similar to that reported for the vaginal and oral mucosa of women after microbiological culture (Srinivasan *et al.*, 2009), and contained predominantly lactobacilli, and several other prevalent bacterial species including *Bifidobacterium* spp., *Staphylococcus* spp., *Actinomyces* spp., *Propionibacterium* spp., *Peptostreptococcus* spp. , *Streptococcus* spp. , and enteric bacteria, were also detected. We acknowledge that the vagina may not be the only source of the contaminating microorganisms detected in the follicular fluids tested in this study; however, our definition worked within the limitations of the oocyte retrieval technique (trans-vaginal) routinely employed at Wesley Monash IVF, therefore, we have reported 'colonised' follicular fluid only when the same microbial species were not also detected in parallel testing of the vaginal secretions.

In addition to identifying microorganisms present within follicular fluid, this study concluded that colonisation was not related to the primary cause of infertility. Pathogenic bacteria were identified as colonisers of follicular fluid, in both fertile and infertile women, suggesting that colonising microorganisms in follicular fluid

were not restricted to infertile women. This finding may explain the conservative natural conception rate, which remains around 30% per ovulatory cycle (te Velde and Cohlen, 1999). Follicular fluid colonisation by opportunistic or pathogenic microorganisms may also be an explanation for the reproductive health issues experienced by couples diagnosed with idiopathic infertility. Moreover, microorganisms colonising follicular fluid may be transferred to the uterine tubes, the uterus or the peritoneal cavity following (i) ovulation in natural cycles, or (ii) ovarian puncture for IVF. This may lead to latent infections such as salpingitis, PID or tubo-ovarian abscesses (Bennett *et al.*, 1993, Sharpe *et al.*, 2006, Tureck *et al.*, 1993) or acute infection during pregnancy (which represents an immunocompromised state) or post-partum.

The oocyte retrieval procedure may puncture an *in vivo* ovarian follicular fluid biofilm, resulting in translocation of a biofilm portion to another site where this smaller community can begin to establish a new biofilm. Identification of the microbial species present in follicular fluid in infertile women who have repeated failed IVF attempts or early pregnancy losses may inform antimicrobial treatment and lead to improved outcomes in subsequent cycles. The translocation of a follicular fluid biofilm may result in different reproductive outcomes compared to the translocation of planktonic bacteria, because of the altered growth characteristics observed in sessile bacteria. The controlled and often low levels of metabolic activity of sessile bacteria within biofilms may result in persistence of some species and long-term activation of the host immune response. This type of growth may lead to alterations in the genital tract secretions in response to changes in the host cytokine expression or molecular signals between species within the biofilm.

Interestingly, in this study, a greater diversity of microorganisms was isolated from the follicular fluids collected from the left ovary than from the right ovary. This was an interesting observation since literature reports asymmetry between the vasculature draining the left and right ovaries (Last, 1984). Additionally, throughout the reproductive lifetime, in naturally cycling women, ovulation occurs more frequently in the right ovary than the left ovary (Potashnik *et al*., 1987, Fukuda *et al*., 2000). In women undergoing IVF, the response to exogenous hormones is reportedly more elevated in the right ovary, compared to the left ovary (Lan *et al.*, 2010). Furthermore, an increased number of oocytes are retrieved from the right ovary and embryos formed following fertilisation, as well as the development of superior quality embryos, compared to those formed from oocytes collected from the left ovary (Lan *et al*., 2010). However, despite these observations, previous studies have concluded that the origin of the oocytes (left or right ovary) did not affect the implantation or pregnancy rates (Lan *et al*., 2010, Fukuda *et al*., 2000), but taken together these findings suggest that the follicular environment plays a significant role in oocyte and early embryo quality.

Further highlighting the significance of ovarian asymmetry, investigations of ovarian cancer patients revealed that metastatic disease and a poor prognosis was more frequently associated with a right sided primary ovarian cancer, than a left ovarian cancer, as a result of the differences in vasculature and pelvic lymph nodes (Dane *et al.*, 2008). Together with the observations above, our data suggest that it would be worthwhile to evaluate further the microflora of the left and right follicular fluids to determine whether this also results in differences in IVF outcomes.

Infection and the associated local inflammation occur when the homeostatic balance between the host, environment and microorganisms is disrupted (Salcido, 2007). Despite microbial loads ranging from 10^3 CFU / mL to $>10^6$ CFU / mL, no follicular fluid specimens in this study appeared visibly turbid, however visible turbidity is observed in other body fluids as a result if these same infection rates and the presence of white blood cells and bacteria. This suggests that there was little or no alteration in the immune response to the presence of microorganisms within follicular fluid possibly due to (1) non-replicating or slow growing microorganisms or (2) the presence of microorganisms in a protected growth phenotype (dormant or biofilm). These data suggested that the microbial load alone was not the major contributing factor in adverse IVF outcomes. Infectious disease studies have previously reported that the number of microorganisms present, microbial virulence factors and host resistance combined, determine the extent of adverse outcomes associated with microorganisms (Edwards and Harding, 2004).

A study of the pathogenesis of the diverse bacteria within follicular fluid revealed an interesting and significant finding, that microorganisms within follicular fluid formed biofilms *in vitro*. Biofilms are ubiquitous and associated with up to 80% of serious infections in humans (Harro *et al.*, 2010). Mucosal sites in humans are protected by biofilms comprising the normal regional microflora and cytokines. These sites serve to prime the immune response, such that there is a level of tolerance to the presence of the normal microflora. How do microorganisms persist at an anatomical site believed to be 'sterile' or only transiently colonise a postulated hostile environment? Many studies have concluded that the difference between acute and chronic infections is the formation of a biofilm (Furukawa *et al.*, 2006). A biofilm leads to

resistance to not only assault by the host immune response and against antimicrobial therapy but also against opportunistic infection by other microorganisms (Siddiqui and Bernstein, 2010, Stewart and Costerton, 2001).

Microbial species detected in follicular fluid developed into mature biofilms over a period of 10 days. In addition, it was demonstrated that viable microorganisms may be recovered from follicular fluid incubated at 37° C aerobically for a period of 28 weeks. The fact that microorganisms can survive in a small amount of follicular fluid without nutritional replenishment or waste removal highlights the potential of follicular fluid as a microbial growth medium. The ability of microorganisms to persist for prolonged periods in follicular fluid has serious implications for all aspects of reproduction. Follicular fluid microorganisms may damage the maturing oocyte, be transported to the uterus at the time of ovulation, elicit an immune response causing inflammation and/or scar tissue in the genital tract or infect the developing foetus, potentially leading to neonatal and /or maternal morbidity or mortality.

Follicular fluid contains a low level of dissolved oxygen and in this study we isolated a greater number of anaerobes (70%) than aerobes (30%) from follicular fluid culture. The concept of colonisation has been well established in studies investigating wound microflora (Siddiqui and Bernstein, 2010). These studies perhaps offer a model for understanding the potential impact of microbial colonisation of human follicular fluid. Wounds represent a hypoxic environment, often dominated by anaerobic microflora (Landis, 2008). Wound studies have revealed that the microflora changes throughout the life of the wound and relative to

microbial synergism. Data from this thesis confirmed that like many other anatomical sites, the follicular fluid microflora was not static. In the long-term *in vitro* culture experiments, the microorganisms changed from polymicrobial to monomicrobial over time.

Follicular fluid itself contains many components, which can act as substrates for essential cellular processes including glycolysis (Edwards, 1974). Bacteria may metabolise follicular fluid macromolecules (carbohydrates, cholesterol etc), producing by-products such as carbon dioxide, a key metabolite product (Wintermute and Silver, 2010 a, b). Physiologically, carbon dioxide exists in equilibrium with bicarbonate through the enzymatic action of carbonic anhydrase, an enzyme present both in human cells and in bacterial cells (Smith and Ferry, 2000). Therefore, *in vivo* bacterial metabolism in follicular fluid may result in alterations in the carbon dioxide and bicarbonate levels, which would alter the pH and buffering capacity of follicular fluid, potentially causing oxidative stress to the oocyte. Furthermore, the bicarbonate ion also ensures that the granulosa cells are separated both from each other and from the zona pellucida (Zamboni *et al.*, 1965). Following ovulation, the tubal secretions contain significant levels of carbonic anhydrase, required for the disruption of the granulosa cells and zona pellucida to allow fertilisation by a spermatozoon (Mastroianni, 1999). The presence of elevated levels of bacterial carbonic anhydrase in follicular fluid may also result in early atresia of the oocyte if the cumulus cells and zona pellucida are degraded, and fertilisation does not rapidly follow. Successful *in vitro* manipulation of oocytes, sperm and embryos has depended on the development of appropriate culture media. Carbon dioxide equilibration of media to produce optimal pH and bicarbonate buffering (for use outside the carbon dioxide

incubator) has enabled the necessary manipulation of gametes and embryos in IVF. Sessile bacteria within biofilms behave in a very different manner to planktonic bacterial cells, through changes to gene regulation (Jakubovics, 2010, Jakubovics and Kolenbrander, 2010, Lopez *et al.*, 2010). Bacterial metabolites within follicular fluid *in vivo* present two possibilities: (1) bacteria and their metabolic by-products are detrimental to oocyte quality, through depletion of nutrients including but not limited to glucose, pyruvte and lactate and/or production of excess carbon dioxide or bicarbonate, which can result in a damaging pH shift, or (2) 'slow growing'/dormant microorganisms, which only minimally deplete nutrients within follicular fluid causing changes in the pH, which are successfully compensated by homeostatic buffering within the follicular environment, resulting in no adverse effects or a potentially beneficial effect.

While many bacterial species (ranging from aerobic, members of the Enterobacteriaceae, *Staphylococcus* spp. and *Streptococcus* spp. to anaerobic species including *Bacteroides* spp., *Peptostreptococcus* spp. and *Prevotella* spp.) recovered from follicular fluid were associated with adverse IVF outcomes, *Lactobacillus* spp. were associated with successful outcomes. These findings may be related to the metabolic activity of each individual microbial species. For example, the production of reactive oxygen species has been associated with oxidative stress and poor oocyte quality (Tamura *et al.*, 2008). For example, we reported that *Lactobacillus* spp. formed mature biofilms *in vitro* and if this also occurs *in vivo*, then it may result in the slower growth and metabolic activity routinely associated with bacteria in biofilms (Zur *et al.*, 2004). Previous studies have reported that *Lactobacillus* spp.

biofilms can also offer protection against opportunistic infection by displacing other bacterial cells or biofilms (Patterson *et al.*, 2009).

A previous wound study also concluded that microbial colonisation may enhance wound healing through local inflammation and increased perfusion (Laato *et al.*, 1988). In this current study, we also found that follicular fluid levels of VEGF, a potent angiogenic cytokine were elevated in the presence of colonising bacteria. This supports the hypothesis that the intimate relationship between the constant remodelling of the ovarian vasculature, (programmed by the body for normal menstrual cycling) inadvertently results in enhanced transport and survival of microorganisms in this anatomical niche. Alternatively, the elevated VEGF may represent an immune response, an attempt to increase perfusion and thus immune cell numbers within the ovary in response to the damage caused within the ovarian follicles by microorganisms.

In this current project, it was also demonstrated that the steroid hormones, oestradiol and progesterone (in culture media in concentrations achieved in the follicular fluid from women undergoing IVF) were capable of modulating the growth *in vitro* of microorganisms isolated from follicular fluid. Therefore, fluctuations in genital tract hormone levels, as part of the menstrual cycle, or the establishment of a pregnancy, may provide nutrients that allow microorganisms in follicular fluid or the UGT to proliferate. Previous studies have confirmed that there is a relationship between the prevalence of microbial species and the stages of the menstrual cycle. It has been concluded that many infections appear to be steroid hormone dependent (Sonnex, 1998). Women undergoing ovarian hyperstimulation for trans-vaginal oocyte

retrieval and IVF share the same menstrual cycle pattern as women who conceive naturally, however the genital tract steroid hormone concentrations in these women are significantly elevated due to the increased number of mature ovarian follicles, which stimulate hormone production. This may suggest that microorganisms that are particularly sensitive to steroid hormones as growth factors or inhibitors would either be enhanced or reduced in women who conceive during the stimulated cycle. In subsequent cycles, where frozen embryos are transferred and the menstrual cycle more closely mimics that of fertile women, when only a single dominant follicle exists, then the microflora may be similar between the two groups.

One bacterial species that was not dependent on hormones for *in vitro* growth was *E. coli*. In this study, *E. coli* isolated from follicular fluid did not form biofilms. The hormonal stimulation protocols used for the maturation of multiple oocytes for IVF may contribute to the modulation of the follicular fluid microflora *in vivo* by promoting or inhibiting microbial growth of hormone-dependent species. A high level of oestradiol reportedly inhibits the attachment of *E. coli* to cells *in vitro* and may explain the increased isolation of *E. coli* from the genital tracts of post-menopausal women, who are not receiving treatment with exogenous oestrogen hormone replacement therapy (Raz, 1993). Similarly, genital tract infections caused by some microbial species are more frequently diagnosed at specific stages of the menstrual cycle (Sonnex, 1998). High levels of progesterone are secreted from the corpus luteum following ovulation to maintain the uterine environment in readiness for pregnancy. In IVF, exogenous progesterone is prescribed as luteal support and may promote delayed regression of the corpus luteum, potentially maintaining microorganisms within the follicle for weeks. Microorganisms that either utilise, or

are inhibited by oestradiol or progesterone may be particularly susceptible to the follicular fluid environment (*Lactobacillus* spp., *Bacteroides* spp., *E. coli*).

In this study, cytokine profiles were identified, which may be useful for predicting whether IVF fertilisation will occur and/or whether subsequent embryo transfer will be successful. Increased levels of IL-1 α, IL-1 β and VEGF (Table 6.1) in vaginal secretions were associated with successful fertilisation, and may potentially be used to predict this outcome. A follicular fluid cytokine profile (elevated levels of IL-6, IL-12p40, GCSF and IFN γ) (Table 6.1) was also shown to be associated with successful embryo transfer, suggesting its potential in predicting embryo transfer. These findings further support the use of cytokine detection to monitor treatment and predict IVF outcomes.

Semen primes the genital tract, stimulating an inflammatory response necessary for successful fertilisation and implantation (Robertson *et al.*, 2009). This may explain why a vaginal cytokine profile at the time of hCG induced ovulation may predict fertilisation outcomes. The ability to predict successful fertilisation using a vaginal swab offers a non-invasive screening tool, which clinicians may potentially use for women trying to conceive naturally or prior to commencement of an IVF cycle. The result may aid in determining, which couples would need/benefit from IVF or more specifically ICSI fertilisation to conceive. In contrast, successful embryo transfer was correlated to a follicular fluid cytokine profile, which may suggest that the follicular environment should be further evaluated with respect to the impact on oocyte quality and embryos.

Currently, the IVF scientists select the embryo that has the best morphological grading for transfer to the uterus in each IVF treatment cycle (Boiso *et al.*, 2002). Additional information available regarding the chance of successful transfer for any given embryo may reduce the number of failed cycles. In this study, we have identified cytokine profiles associated with successful fertilisation and successful embryo transfer. Therefore, based on a cytokine profile of follicular fluid and/or vaginal secretions, the clinician may be able to provide counselling to patients in cases where the outcome is likely to be negative (failed fertilisation or failed embryo transfer) so that they can make an informed decision about whether/or how to proceed.

This is the first study to identify a single cytokine, IL-18 predictive of idiopathic infertility. This finding warrants further investigation and may be useful in determining the pathogenesis of idiopathic infertility. The cytokine response for females with idiopathic infertility, as expected was site-specific, differing between the follicular fluid and vaginal secretions. What was surprising was that the presence of microorganisms in follicular fluid did not result in the production of cytokines characteristic of an overt pro-inflammatory response (Dinarello, 2000). Future studies should focus on specific cytokine profiles produced in response to bacterial species at each of these genital tract sites to correlate IVF outcomes with genital tract flora and the immune response.

'Fertile' women (the cohort with infertile male partners) did not have (1) lower levels of follicular fluid colonisation (27%) compared to infertile women (29%), or (2) less adverse ART outcomes compared to infertile women with colonising bacteria in

follicular fluid. What is the natural reservoir of colonising species? The high levels of VEGF associated with follicular fluid colonisation would support haematogenous dissemination and seeding of microorganisms from other anatomical sites. Oral and vaginal microflora from the female and oral flora from the male partner have been isolated from chorioamnionitis cases (Srinivasan *et al.*, 2009). Furthermore, respiratory tract infection and gastrointestinal tract flora have also been isolated from tubo-ovarian abscesses (Goulet *et al.*, 1995b, Hartmann *et al.*, 2009, Kepkep *et al.*, 2006). Something as simple as brushing one's teeth has been associated with adverse pregnancy outcomes including pre-term birth (Han, 2011). Due to the extensive ovarian vasculature, the microorganisms detected within the follicular fluid may travel from anywhere in the body. The follicular fluid microflora can affect the developing oocyte prior to collection for IVF; and have potentially devastating effects on fertility, causing damage to oocytes and resulting in poor quality embryos and pregnancy loss or complications. **It is time to consider asymptomatic microbial colonisers of follicular fluid as contributors to adverse IVF outcomes.**

The novel findings reported in this thesis, demonstrated that follicular fluid contains a diverse range of microorganisms and a site-specific cytokine profile, which were associated with adverse IVF outcomes offers hope to infertile couples. A better understanding of the individual microflora of the UGT and their effect on oocytes may lead to earlier intervention by clinicians, the refinement of fertility treatment and a reduction in the impact of microorganisms on IVF outcomes. Identification and control of deleterious microorganisms within follicular fluid may reduce the need for IVF for some couples.

7.3 Future directions

What remains to be established is the effect of the presence of the microbial species detected in follicular fluid on oocyte quality. The cumulus oocyte complex is bathed in follicular fluid for an extended period (at least 7-10 days) during *in vivo* maturation. Microorganisms within a follicle or their toxins (hyaluronidases, proteases, lipases) and/or their metabolites, may affect the oocyte itself, the oocyte DNA or the cumulus cells that supply nutrition to the oocyte. Furthermore, we have shown that gentamicin, the antimicrobial routinely used in IVF culture media, does not affect a number of the species (*Corynebacterium* spp. and anaerobes) that have been isolated from follicular fluid (Results not shown). Antimicrobial prophylaxes are administered to some women (depending on the clinician) at the time of trans-vaginal oocyte retrieval. Whilst this may prevent post-operative infection, this treatment would be too late to prevent damage to the oocytes, which have already matured within the colonised *in vivo* environment. The results of this study have shown that the presence of colonising microorganisms with follicular fluid was associated with adverse IVF outcomes. Oocytes bathed in microorganisms and their metabolites may result in poor quality embryos, low implantation rates, early pregnancy losses or preterm birth.

The knowledge that we now have, that follicular fluid can be colonised by a diverse range of microorganisms may lead to routine screening of follicular fluid and, if warranted, antimicrobial treatment of women with repeated failed IVF attempts prior to subsequent trans-vaginal oocyte retrievals. Furthermore, an understanding of the level of biofilm formation and persistence may lead to further research into probiotic supplements to allow disruption of pathogenic biofilms associated with adverse IVF

outcomes. Supplemental probiotic *Lactobacillus* spp. has been trialled in IVF patients with no positive improvements; however, the species were not the prevalent genital tract species, but instead were those more predominant within the gastrointestinal tract. A previous study treating women with bacterial vaginosis with probiotic *Lactobacillus* spp. (*L.iners*, a species infrequently reported as genital tract normal regional flora), did report reversion of the vaginal flora to a healthy *Lactobacillus* spp. dominant form following treatment. This suggests that probiotics require further investigation as a potential non-invasive primary treatment or adjuvant to traditional therapy.

Based on the results of this study the development of a suite of real-time PCR assays to identify rapidly the most prevalent species detected within follicular fluid will be developed. This information will assist clinicians in the management of IVF patients. This area of research should now also focus on the characterisation of the virulence mechanisms of the most prevalent microorganisms isolated from follicular fluid in order to more adequately understand how individual, or combinations of species can lead to adverse IVF outcomes, including decreased fertilisation, poor quality embryos or early pregnancy losses.

Intravenous immunotherapy has reportedly resulted in the successful treatment of infertile women with repeated spontaneous miscarriage or anti-sperm antibodies (Scarpellini and Sbracia, 2009, Toth *et al.*, 2010). The identification of altered cytokine levels within follicular fluid, related to adverse IVF outcomes may offer another opportunity for clinical intervention. Macrophage polarisation assays performed on cells isolated from follicular fluid, would be useful to determine

whether the cytokine response is the 'normal' inflammatory response associated with ovulation and the menstrual cycle, or whether cells recruited to the follicular fluid are there in response to the microorganisms. Animal studies using knock-out models or intravenous injections of cytokines identified in this thesis may be useful for investigating the potential of immunomodulation, during either folliculogenesis or during the pre-implantation period. In addition, polarisation assays may identify further targets. The exogenous introduction of either cytokines or immunoglobulins into the systemic circulation may provide rapid and effective treatment for women with an inappropriate localised immune response.

Metabolomics is a rapidly growing area of research. Metabolomic profiles have recently been published for follicular fluid and also embryos in culture (Katz *et al.*, 1992, Pinero-Sagredo *et al.*, 2010). Investigations of *in vivo* culture media in relation to the follicular fluid microflora may be useful in determining, which microbial species result in significant alteration to key biological components such as glucose, pyruvate, lactate and other members of the carbon cycle.

At Wesley Monash IVF, all oocyte retrievals are performed per vagina. We note that the sampling method may introduce microorganisms into the follicular fluid at the time of oocyte collection. In this current study, only the follicular fluids from the largest most accessible follicles in both the left and/or right ovary were cultured to detect microorganisms. However, more recently in our laboratory, we have cultured the separately pooled follicular fluids collected from all follicles in the left and right ovaries and we have demonstrated that for 35% of women, the microorganisms present within the largest follicle are the same as those collected from other follicles

within the same ovary (J. Harris, Personal communication.) This further supports the proposal that the adverse outcomes demonstrated in this thesis are due the presence of colonising bacteria within the follicular fluid. We would suggest that in future studies, collection of follicular fluid at the time of operative laparoscopy would be useful to confirm the findings reported in this study.

Further information regarding the novel findings from this project, the follicular fluid microflora and its impact on IVF outcomes may result in (1) the selection of the most appropriate ART treatment protocols for couples, (2) targeted prophylactic treatment prior to oocyte retrieval, (3) changes within the work flow of the ART laboratory, (4) tracking of oocytes from the left and right ovary, and (5) correlations of conventional morphological analyses /selection of oocytes with the presence of colonising bacteria.

This study has extended our knowledge of microflora and cytokines the follicular fluid in fertile and infertile women undergoing IVF treatment cycles. We have shown that the collection of follicular fluid, a specimen that is routinely discarded as part of an IVF cycle offers a significant opportunity to address IVF failures.

CHAPTER EIGHT

APPENDICES

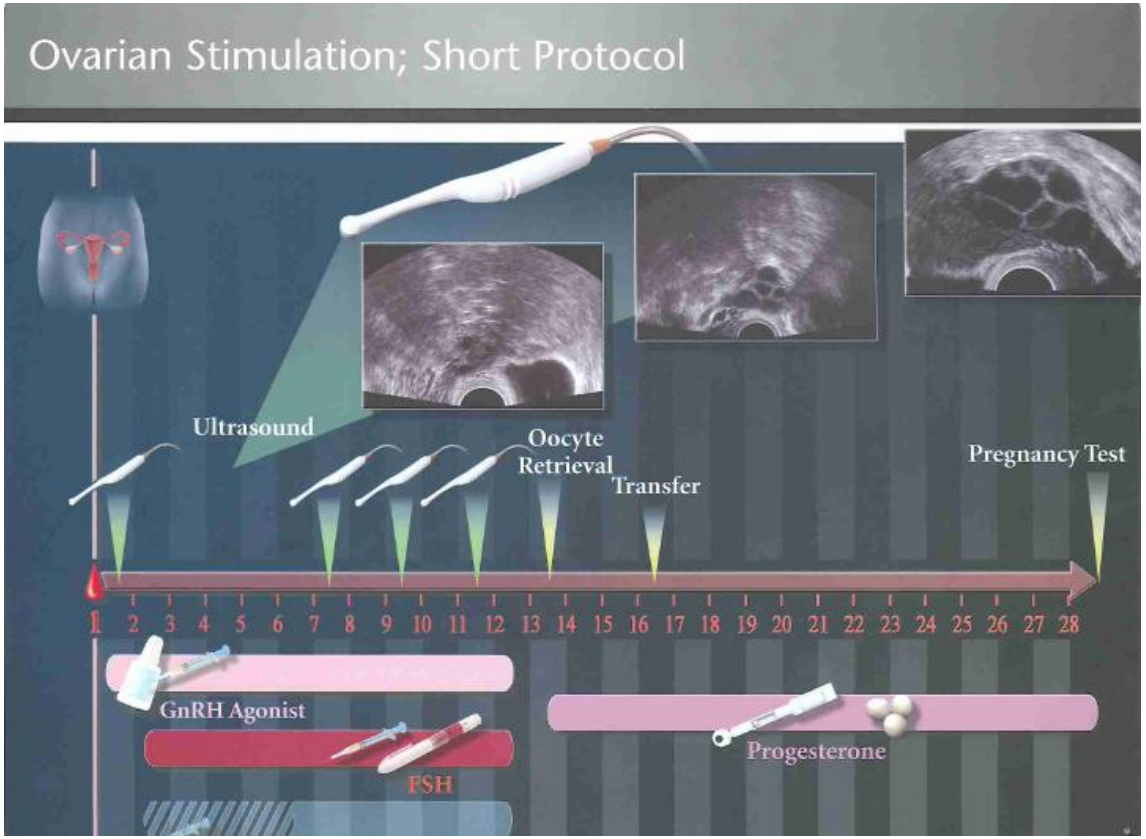
Appendix 1. Host defense proteins expressed in the male reproductive tract

(Adapted from Hook *et al*., 2002)

Host defense proteins expressed in the male reproductive tract

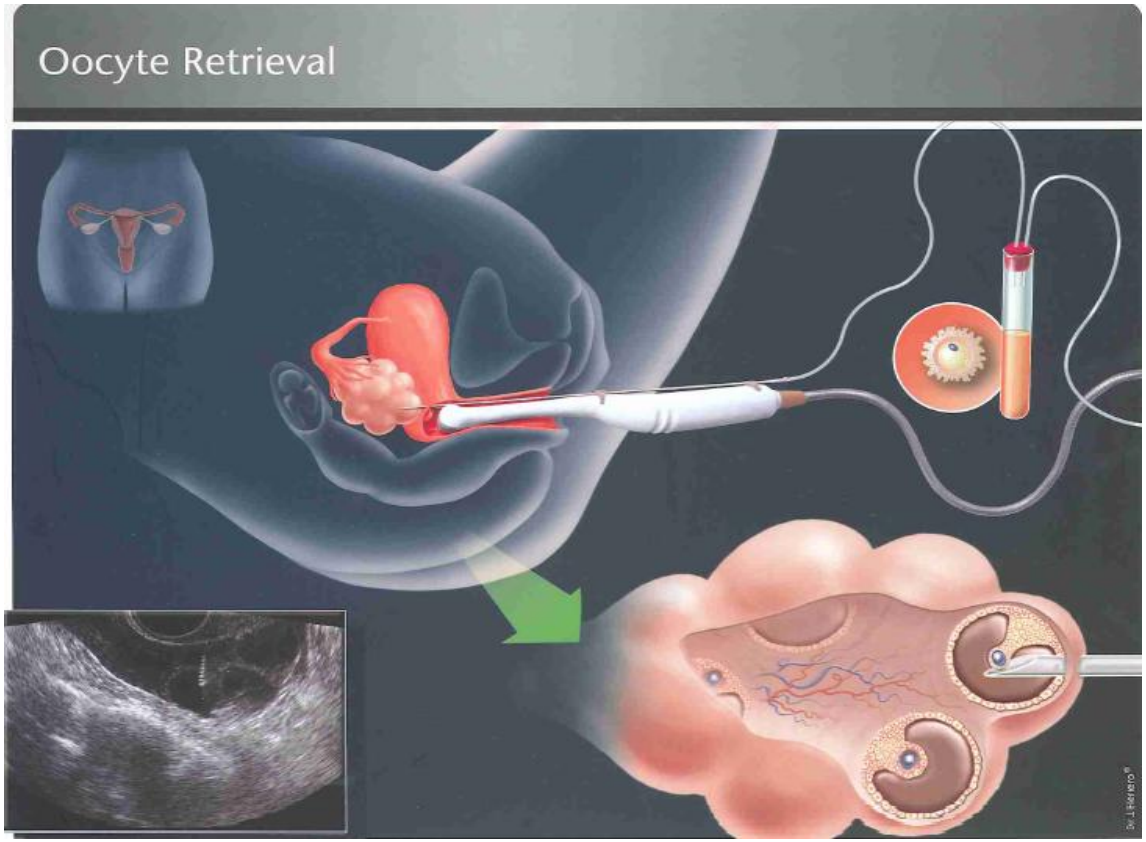
Protein	Sites of Expression	Functions	
		Known	Proposed
Mucins	Testis epididymis, prostate	Mobile barrier to prevent entry of bacteria, viruses, toxins	Bind and remove pathogens from the male reproductive tract
β-Defensins	Testis, epididymis, prostate, seminal vesicle	Kill *Staphylococcus, Enterococcus, Escherichia coli, Candida*; chemotaxis	Sperm and epithelial protection
Cathelicidins	Epididymis, neutrophils	Kill *Staphylococcus, E. coli, Pseudomonas*; chemotaxis, sperm-binding	Epithelial cell protection; mobile defense system in male and female reproductive tracts
Bin1b/HE2	Epididymis	Kill *E. coli*; sperm binding	Antimicrobial protection of sperm in male and female tracts
α_2-Macroglobulin	Sertoli cells	Nonspecific inhibition of mammalian proteases	Inhibition of microbial proteases
Secretory leukocyte protease inhibitor	Epididymis, prostate, seminal vesicles	Inhibition of elastase, cathepsin G, and proteases of *Aspergillis*; inhibition of viral infection, kill *E. coli* and *Staphylococcus*	Antimicrobial action in the male reproductive tract
Cystatin C	Sertoli and germ cells, epididymis, prostate, seminal vesicles	Inhibition of mammalian cathepsins, inhibition of group A streptococcal cysteine protease	Antimicrobial action in the male tract; inhibition of microbial proteases
Male reproductive cystatins	Testis, epididymis	Unknown	Antimicrobial action in the male reproductive tract
Tear lipocalin	Germ cells, prostate	Bind and transport small hydrophobic molecules	Host defense in the male reproductive tract
Lung surfactant Protein D (SP-D)	Testis, prostate	Limit lung infection by *E. coli Salmonella, Haemophilus, Pseudomonas, Pneumocystis, Aspergillus*; chemotactic for phagocytes	Protection of the male tract against bacteria and fungi andrecruitment of phagocytes
Lactoferrin	Epididymis	Nutrient sequestering inhibits infection by bacteria, fungi, and viruses	Antimicrobial protection of sperm in male and female tracts
Lysozyme	Testis, epididymis, prostate	Bacterial lysis	Bacterial lysis in the male tract
Toll-like receptors	Testis, prostate	Cell surface mediators of intracellular responses to pathogens	Mediate induction of antimicrobial peptides in response to microbial invasion
NOD1, NOD2	Testis	Intracellular pathogen recognition receptors	Mediate induction of defense pathways in response to viral, bacterial and eukaryotic pathogens

Appendix 2. Diagram outlining 'flare' hormonal stimulation protocol for trans-vaginal oocyte retrieval procedure and embryo transfer



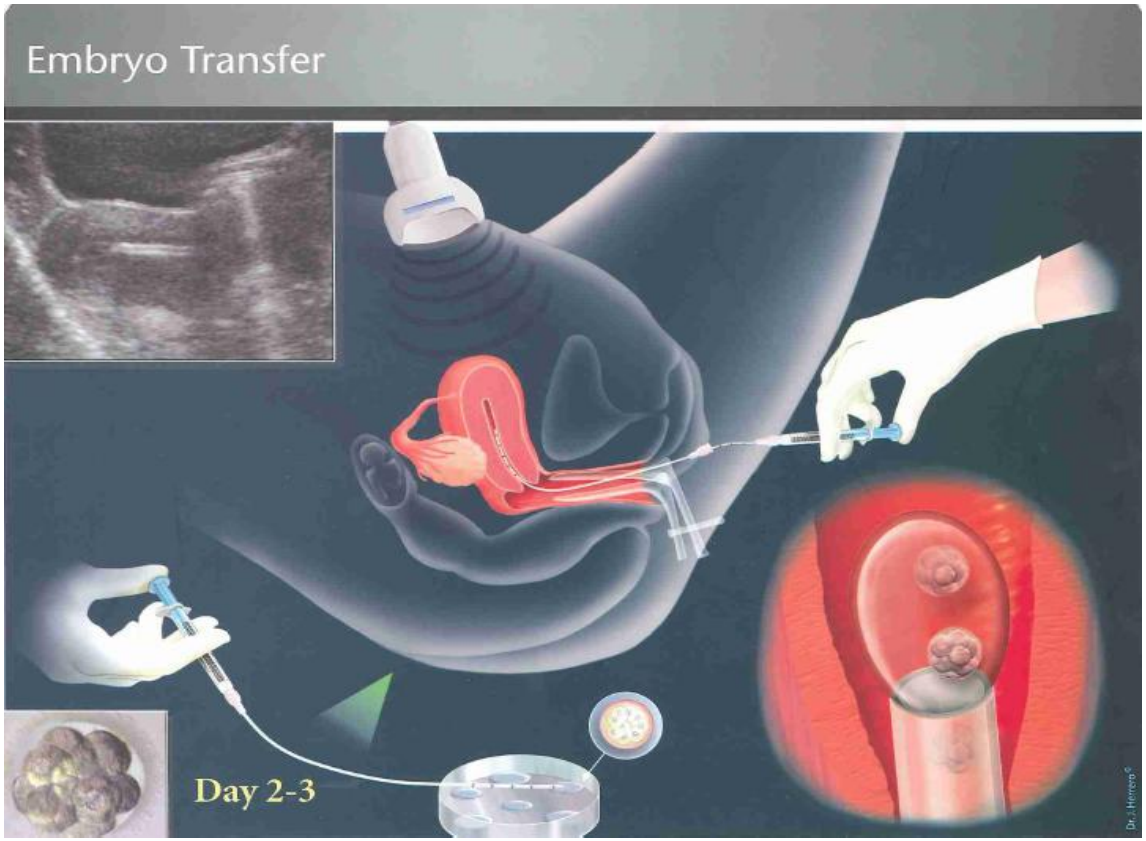
(Serono International, 2006)

Appendix 3. Diagram outlining the trans-vaginal oocyte retrieval procedure



(Serono International, 2006)

Appendix 4. Diagram outlining the embryo transfer procedure



(Serono International, 2006)

Appendix 5. Microbiological culture data for the cytokine sub-cohort of 71 women

	Colonised			**Contaminated**			**Male factor (fertile women)**			**Endometriosis**			**Polycystic ovary syndrome**			**Genital infection**			**Idiopathic**		
Microbial species	Left Ovary (L)	Right Ovary (R)	Vagina (V)	Left	Right	Vagina	L	R	V	L	R	V	L	R	V	L	R	V	L	R	V
***Actinomyces* species**	+	+	-	+	+	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	-
A. israelii	-	+	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-
	-	-	+	+	+	+	-	-	+	-	-	-	-	-	+	+	-	-	-	-	+
A. naeslundii	+	-	-	+	-	+	+	+	+	+	-	+	+	+	+	+	-	+	+	-	-
***Bacteroides* spp.**	-	-	+	+	+	+	-	-	-	+	-	+	-	-	-	-	-	+	-	-	+
***Bifidobacterium* spp. 1**	-	-	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
***Bifidobacterium* spp. 2**	-	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+

Candida albicans	-	-	+	+	-	+	-	-	+	-	-	+	+	-	+	+	-	+	-	-	-
C. glabrata	-	-	+	+	+	+	-	-	+	-	+	+	-	-	-	+	-	-	+	+	+
C. parapsilosis	+	-	-	+	-	+	-	-	-	+	-	-	-	-	-	+	+	+	-	-	+
Clostridium butyricum	-	-	+	+	-	+	+	-	+	-	-	-	+	-	+	+	-	+	-	+	+
C. ramosum	-	-	+	+	-	+	-	-	+	-	-	+	-	-	+	+	-	+	-	-	-
***Corynebacterium* spp.**	-	-	+	+	+	+	-	-	+	-	-	-	-	+	+	+	-	+	-	-	-
C. auromucosum	+	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	+	-	-	+
Escherichia coli	-	-	+	+	-	+	-	-	+	-	-	+	-	-	+	+	+	+	-	-	+
Enterococcus faecalis	-	-	+	+	-	+	-	-	+	-	-	-	-	+	+	+	-	+	-	-	+
Egghertella lenta	-	-	+	+	-	+	-	-	+	-	-	-	-	-	+	+	-	+	+	-	+
***Fusobacterium* spp.**	+	-	-	-	-	+	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-
***Gemella* spp.**	-	-	+	+	-	+	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-
***Lactobacillus* spp.**	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L. crispatus	-	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+

L. gasseri	-	-	+	+	+	+	+	-	+	+	-	-	+	-	+	+	+	+	+	+	+
L. iners	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
L. jensenii	-	-	+	+	+	+	-	-	+	-	+	+	+	-	-	+	+	+	+	+	+
Propionibacterium acnes	-	-	+	+	+	+	-	+	+	+	-	+	-	+	+	+	+	+	+	+	+
P. avidum	+	-	-	+	-	+	-	-	+	-	-	-	-	-	+	+	-	-	-	-	+
P. granulosum	+	+	-	-	-	-	+	+	-	-	-	-	-	+	-	+	-	-	-	-	-
P. propionicus	+	+	-	+	+	+	-	-	+	-	-	+	-	-	+	+	+	+	+	+	-
Prevotella disiens	+	-	-	-	-	+	-	-	+	-	-	-	-	-	+	+	-	-	-	-	-
P. melanogenicus	+	-	-	+	-	+	-	-	+	-	-	-	+	-	+	+	-	-	-	-	+
Peptinophilus asaccharolyticus	+	+	-	+	-	+	+	-	+	-	-	-	+	-	+	+	+	-	-	-	-
***Peptostreptococcus* spp.**	+	-	-	-	-	+	+	-	-	-	-	-	+	-	-	+	+	+	+	-	-
***Staphylococcus* spp.**	+	-	-	+	+	+	-	-	+	-	+	+	-	-	+	+	-	+	+	-	-
S. aureus	-	-	-	+	+	+	-	-	+	-	-	+	+	+	+	+	+	+	-	+	+
S. epidermidis	-	-	+	+	+	+	-	-	+	-	-	+	-	-	+	+	+	+	+	+	+
S. lugdunensis	-	-	+	+	+	+	-	-	+	-	-	+	+	+	+	+	+	+	+	-	+

***Streptococcus* spp.**	-	-	+	+	+	+	-	-	+	-	-	-	-	-	+	+	-	-	-	-	-
S. agalactiae	-	-	+	+	+	+	+	+	+	-	-	-	-	-	-	+	+	+	-	-	+
S. viridans	-	-	+	+	+	+	+	-	+	-	-	-	+	-	+	+	+	+	-	-	+
***Veilonella* spp.**	-	-	-	+	+	+	+	-	+	-	-	-	+	+	+	+	-	+	-	-	-
Total number of different species detected	**14**	**7**	**22**	**33**	**21**	**38**															

CHAPTER NINE

REFERENCES

Abbas, F., Kamal, M. K. and Talati, J. (1995) Prostatic aspergillosis. *J Urol*, **153**, 748-750.

Adashi, E. Y. (1989) Cytokine-mediated regulation of ovarian function: Encounters of a third kind. *Endocrinol*, **124**, 2043-2045.

Aitken, R. J. (1995) Free radicals, lipid peroxidation and sperm function. *Reprod Fertil Dev*, **7**, 659-668.

Aitken, R. J. (1999) The Amoroso lecture. The human spermatozoon-a cell in crisis? *J Reprod Fertil*, **115**, 1-7.

Al-Fozan, H. and Tulandi, T. (2003) Left lateral predisposition of endometriosis and endometrioma. *Obstet Gynecol*, **101**,164-6.

Allison, D. G., Sutherland, I.W. (1984) A staining technique for attached bacteria and its correlation to extracellular carbohydrate production. *J Microbiol Methods*, 93-99.

Alpizar, E. and Spicer, L. J. (1994) Effects of interleukin-6 on proliferation and follicle-stimulating hormone-induced oestradiol production by bovine granulosa cells *in vitro*: Dependence on size of follicle. *Biol Reprod*, **50**, 38-43.

Amato, G., Conte, M., Mazziotti, G., Lalli, E., Vitolo, G., Tucker, A. T., Bellastella, A., Carella, C. and Izzo, A. (2003) Serum and follicular fluid cytokines in polycystic ovary syndrome during stimulated cycles. *Obstet Gynecol*, **101**, 1177-1182.

Amstey, M. S. and Steadman, K. T. (1976) Asymptomatic gonorrhea and pregnancy. *J Am Vener Dis Assoc*, **3**, 14-16.

Andrews, W. W., Goldenberg, R. L. and Hauth, J. C. (1995) Preterm labor: Emerging role of genital tract infections. *Infect Agents Dis*, **4**, 196-211.

Andrews, W. W., Goldenberg, R. L., Hauth, J. C., Cliver, S. P., Conner, M. and Goepfert, A. R. (2005) Endometrial microbial colonisation and plasma cell endometritis after spontaneous or indicated preterm versus term delivery. *Am J Obstet Gynecol*, **193**, 739-745.

Arici, A., Seli, E., Senturk, L. M., Gutierrez, L. S., Oral, E. and Taylor, H. S. (1998) Interleukin-8 in the human endometrium. *J Clin Endocrinol Metab*, **83**, 1783-1787.

Ashworth, M. D., Ross, J. W., Stein, D. R., White, F. J., Desilva, U. W. and Geisert, R. D. (2010) Endometrial caspase-1 and interleukin-18 expression during the oestrous cycle and peri-implantation period of porcine pregnancy and response to early exogenous estrogens administration. *Reprod Biol Endocrinol*, 9, 33-41.

Asimakopoulos, B., Nikolettos, N., Papachristou, D. N., Simopoulou, M., Al-Hasani, S. and Diedrich, K. (2005) Follicular fluid levels of vascular endothelial growth factor and leptin are associated with pregnancy outcome of normal women participating in intracytoplasmic sperm injection cycles. *Physiol Res*, **54**, 263-270.

Asimakopoulos, B., Koster, F., Felberbaum, R., Al-Hasani, S., Diedrich, K. and Nikolettos, N. (2006) Cytokine and hormonal profile in blood serum and follicular fluids during ovarian stimulation with the multidose antagonist or the long agonist protocol. *Hum Reprod*, **21**, 3091-3095.

Asimakopoulos, B., Abu-Hassan, D., Metzen, E., Al-Hasani, S., Diedrich, K. and Nikolettos, N. (2008) The levels of steroid hormones and cytokines in individual follicles are not associated with the fertilisation outcome after intracytoplasmic sperm injection. *Fertil Steril*, **90**, 60-64.

Axemo, P., Ching, C., Machungo, F., Osman, N. B. and Bergstrom, S. (1993) Intrauterine infections and their association with stillbirth and preterm birth in Maputo, Mozambique. *Gynecol Obstet Invest*, **35**, 108-113.

Balasch, J., Martinez-Roman, S., Creus, M., Campo, E., Fortuny, A. and Vanrell., J. (1995) Schistosomiasis: An unusual case of tubal infertility. *Hum Reprod*, **10**, 1725-1727.

Barnhart, K. T., Stolpen, A., Pretorius, E. S. and Malamud, D. (2001) Distribution of a spermicide containing nonoxynol-9 in the vaginal canal and the upper female reproductive tract. *Hum Reprod*, **16**, 1151-1154.

Barron, A. L., Rank, R. G. and Moses, E. B. (1984) Immune response in mice infected in the genital tract with mouse pneumonitis agent (*Chlamydia trachomatis* biovar). *Infect Immun*, **44**, 82-85.

Barroso, G., Barrionuevo, M., Rao, P., Graham, L., Danforth, D., Huey, S., Abuhamad, A. and Oehninger, S. (1999) Vascular endothelial growth factor, nitric oxide, and leptin follicular fluid levels correlate negatively with embryo quality in IVF patients. *Fertil Steril*, **72**, 1024-1026.

Baseman, J. B., Reddy, S. P. and Dallo, S. F. (1996) Interplay between mycoplasma surface proteins, airway cells, and the protean manifestations of mycoplasma-mediated human infections. *Am J Respir Crit Care Med*, **154**, S137-144.

Benchaib, M., Braun, V., Lornage, J., Hadj, S., Salle, B., Lejeune, H. and Guerin, J. F. (2003) Sperm DNA fragmentation decreases the pregnancy rate in an assisted reproductive technique. *Hum Reprod*, **18**, 1023-1028.

Bennett, S., Waterstone, J., Cheng, W. and Parsons, J. (1993) Complications of trans-vaginal ultrasound-directed follicle aspiration: A review of 2670 consecutive procedures. *J Assist Reprod Genetics*, **10**, 72-77.

Berger, R. (1999) Acute epididymitis. In Holmes, K. K. *et al*. (eds.) *Sexually Transmitted Diseases*. McGraw-Hill, New York.

Bergh, T. and Lundvisk, O. (1992) Clinical complications during *in vitro* fertilisation treatment. *Hum Reprod*, **7**, 625-626.

Bertrand, E., Zissis, G., Marissens, D., Gerard, M., Rozenberg, S., Barlow, P. (2004) Presence of HIV-1 in follicular fluids, flushes and cumulus oophorus cells of HIV-1-seropositive women during assisted-reproduction technology. *Aids, **18***, 823-825.

Bessen, D. and Gotschlich, E. C. (1986) Interactions of gonococci with hela cells: Attachment, detachment, replication, penetration, and the role of protein II. *Infect Immun*, **54**, 154-160.

Bezold, G., Politch, J.A., Kiviat, N.B., Kuypers, J.M., Wolff, H., and Anderson, D.J. (2007) Prevalence of sexually transmissible pathogens in semen from asymptomatic male infertility patients with and without leukocytospermia. *Fertil Steril*, **87**, 1087-1097.

Bland, K. G. and Gelfand, M. (1970) The effects of schistosomiasis on the Fallopian tubes in the African female. *J Obstet Gynaecol Br Commonw*, **77**, 1024-1027.

Boiso, I., Veiga, A. and Edwards, R. G. (2002) Fundamentals of human embryonic growth *in vitro* and the selection of high-quality embryos for transfer. *Reprod Biomed Online*, **5**, 328-350.

Borini, A., Tarozzi, N., Bizzaro, D., Bonu, M. A., Fava, L., Flamigni, C. and Coticchio, G. (2006) Sperm DNA fragmentation: Paternal effect on early post-implantation embryo development in art. *Hum Reprod*, **21**, 2876-2881.

Bourgeon, F., Evrard, B., Brillard-Bourdet, M., Colleu, D., Jegou, B. and Pineau, C. (2004) Involvement of semenogelin-derived peptides in the antibacterial activity of human seminal plasma. *Biol Reprod*, **70**, 768-774.

Brabin, L., Fairbrother, E., Mandal, D., Roberts, S. A., Higgins, S. P., Chandiok, S., Wood, P., Barnard, G. and Kitchener, H. C. (2005 a) Biological and hormonal markers of chlamydia, human papillomavirus, and bacterial vaginosis among adolescents attending genitourinary medicine clinics. *Sex Transm Infect*, **81**, 128-132.

Brabin, L., Roberts, S. A., Fairbrother, E., Mandal, D., Higgins, S. P., Chandiok, S., Wood, P., Barnard, G. and Kitchener, H. C. (2005 b) Factors affecting vaginal pH levels among female adolescents attending genitourinary medicine clinics. *Sex Transm Infect*, **81**, 483-487.

Brannstrom, M., Mayrhofer, G. and Robertson, S. A. (1993) Localisation of leukocyte subsets in the rat ovary during the periovulatory period. *Biol Reprod*, **48**, 277-286.

Brannstrom, M. and Norman, R. J. (1993) Involvement of leukocytes and cytokines in the ovulatory process and corpus luteum function. *Hum Reprod*, **8**, 1762-1775.

Brannstrom, M., Pascoe, V., Norman, R. J. and McClure, N. (1994) Localisation of leukocyte subsets in the follicle wall and in the corpus luteum throughout the human menstrual cycle. *Fertil Steril*, **61**, 488-495.

Brannstrom, M., Bonello, N., Wang, L. J. and Norman, R. J. (1995) Effects of tumour necrosis factor α (TNF α) on ovulation in the rat ovary. *Reprod Fertil Dev*, **7**, 67-73.

Brunham, R. C. and Peeling, R. W. (1994) *Chlamydia trachomatis* antigens: Role in immunity and pathogenesis. *Infect Agents Dis*, **3**, 218-233.

Buchholz, K. R. and Stephens, R. S. (2006) Activation of the host cell pro-inflammatory interleukin-8 response by *Chlamydia trachomatis*. *Cell Microbiol*, **8**, 1768-79.

Bukulmez, O. and Arici, A. (2000) Leukocytes in ovarian function. *Hum Reprod Update*, **6**, 1-15.

Bungay, S. D., Gentry, P. A. and Gentry, R. D. (2006) Modelling thrombin generation in human ovarian follicular fluid. *Bull Math Biol*, **68**, 2283-302.

Burrello, N., Calogero, A. E., Perdichizzi, A., Salmeri, M., D'Agata, R. and Vicari, E. (2004) Inhibition of oocyte fertilisation by assisted reproductive techniques and increased sperm DNA fragmentation in the presence of *Candida albicans*: A case report. *Reprod Biomed Online*, **8**, 569-573.

Buscher, U., Chen, F. C., Kentenich, H. and Schmiady, H. (1999) Cytokines in the follicular fluid of stimulated and non-stimulated human ovaries; is ovulation a suppressed inflammatory reaction? *Hum Reprod*, **14**, 162-166.

Busey, J. F. (1964) Blastomycosis: I. A review of 198 collected cases in veterans administration hospitals. *Amer Rev Resp Dis*, **89**, 659.

Busolo, F. and Zanchetta, R. (1985) The effect of *Mycoplasma hominis* and *Ureaplasma urealyticum* on hamster egg *in vitro* penetration by human spermatozoa. *Fertil Steril*, **43**, 110-114.

Bussen, S., Zimmermann, M., Schleyer, M. and Steck, T. (1997) Relationship of bacteriological characteristics to semen indices and its influence on fertilisation and pregnancy rates after IVF. *Acta Obstet Gynecol Scand*, **76**, 964-968.

Carey, A. J., Cunningham, K. A., Hafner, L. M., Timms, P. and Beagley, K. W. (2009) Effects of inoculating dose on the kinetics of *Chlamydia muridarum* genital infection in female mice. *Immunol Cell Biol*, **87**, 337-343.

Cassell, G. H., Waites, K. B., Watson, H. L., Crouse, D. T. and Harasawa, R. (1993) *Ureaplasma urealyticum* intrauterine infection: Role in prematurity and disease in newborns. *Clin Microbiol Rev*, **6**, 69-87.

Catlin, B. W. (1992) *Gardnerella vaginalis*: Characteristics, clinical considerations, and controversies. *Clin Microbiol Rev*, **5**, 213-237.

Cauci, S. and Culhane, J. F. (2007) Modulation of vaginal immune response among pregnant women with bacterial vaginosis by *Trichomonas vaginalis*, *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and yeast. *Am J Obstet Gynecol*, **196**, 133.e1-7.

Chalker, V. J., Jordan, K., Ali, T. and Ison, C. (2009) Real-time PCR detection of the mg219 gene of unknown function of *Mycoplasma genitalium* in men with and without non-gonococcal urethritis and their female partners in England. *J Med Microbiol*, **58**, 895-899.

Chan, P. K., Liu, S. J., Cheung, J. L., Cheung, T. H., Yeo, W., Chong, P. and Man, S. (2011) T-cell response to human papillomavirus type 52 L1, E6, and E7 peptides in women with transient infection, cervical intraepithelial neoplasia, and invasive cancer. *J Med Virol*, **83**, 1023-30.

Chen, Y. and Peng, Z. (2000) [Study of oestrogen and progesterone receptors in endometrial carcinoma]. *Hua Xi Yi Ke Da Xue Xue Bao*, **31**, 98-100. Abstract only

Cherpes, T. L., Wiesenfeld, H. C., Melan, M. A., Kant, J. A., Cosentino, L. A., Meyn, L. A. and Hillier, S. L. (2006) The associations between pelvic inflammatory disease, *Trichomonas vaginalis* infection, and positive herpes simplex virus type 2 serology. *Sex Transm Dis*, **33**, 747-752.

Cherpes, T. L., Marrazzo, J. M., Cosentino, L. A., Meyn, L. A., Murray, P. J. and Hillier, S. L. (2008) Hormonal contraceptive use modulates the local inflammatory response to bacterial vaginosis. *Sex Transm Infect*, **84**, 57-61.

Christensen, G. D., Simpson, W.A., Bisno, A.L (1982) Adherence of slime-producing strains of *Staphylococcus epidermidis* to smooth surfaces. *Infect Immunol*, **37**, 318-326.

Chun, S. Y., Eisenhauer, K. M., Kubo, M. and Hsueh, A. J. (1995) Interleukin-1 β suppresses apoptosis in rat ovarian follicles by increasing nitric oxide production. *Endocrinol*, **136**, 3120-3127.

Cicinelli, E., De Ziegler, D., Nicoletti, R., Tinelli, R., Saliani, N., Resta, L., Bellavia, M. and De Vito, D. (2009) Poor reliability of vaginal and endocervical cultures for evaluating microbiology of endometrial cavity in women with chronic endometritis. *Gynecol Obstet Invest*, **68**, 108-115.

Cintron, R. D., Wortham, J. W., Jr. and Acosta, A. (1981) The association of semen factors with the recovery of *Ureaplasma urealyticum*. *Fertil Steril*, **36**, 648-652.

Cochrane, D. M. G. (1988) Immune response to bacterial biofilms. *Med Microbiol J* 255.

Cohen, C., Mugo, N., Astete, S., Odondo, R., Manhart, L., Kiehlbauch, J., Stamm, W., Waiyaki, P., Totten, P. (2005) Detection of *Mycoplasma genitalium* in women with laparoscopically diagnosed acute salpingitis. *Sex Transm Infect*, **81**, 463-466.

Cooper, M. D., Rapp, J., Jeffery-Wiseman, C., Barnes, R. C. and Stephens, D. S. (1990) *Chlamydia trachomatis* infection of human Fallopian tube organ cultures. *J Gen Microbiol*, **136**, 1109-1115.

Corey, L. and Wald, A. (2008) Genital Herpes. In Holmes, K. K. *et al*. (eds.) *Sexually Transmitted Diseases*. McGraw-Hill, New York.

Costerton, J. W. (1999) Introduction to biofilm. *Int J Antimicrob Agents*, **11**, 217-221.

Costerton, J. W., Stewart, P. S. and Greenberg, E. P. (1999) Bacterial biofilms: A common cause of persistent infections. *Science*, **284**, 1318-1322.

Cota, M., Kleinschmidt, A., Ceccherini-Silberstein, F., Aloisi, F., Mengozzi, M., Mantovani, A., Brack-Werner, R. and Poli, G. (2000) Upregulated expression of interleukin-8, RANTES and chemokine receptors in human astrocytic cells infected with HIV-1. *J Neurovirol*, **6**, 75-83.

Cottell, E., McMorrow, J., Lennon, B., Fawsy, M., Cafferkey, M. and Harrison, R. F. (1996) Microbial contamination in an *in vitro* fertilisation-embryo transfer system. *Fertil Steril*, **66**, 776-780.

Craig, M. W., Davey, W. N., Green, R. A. and Plaut, A. (1970) Conjugal blastomycosis. *Am Rev Respir Dis*, **102**, 86-90.

Cunningham, A. L., Diefenbach, R. J., Miranda-Saksena, M., Bosnjak, L., Kim, M., Jones, C. and Douglas, M. W. (2006) The cycle of human herpes simplex virus infection: virus transport and immune control. *J Infect Dis*, **15**, S11-8.

Daly, S. and Prendiville, W. (1997) The management of failed first trimester pregnancy. *Ir Med J*, **90**, 52.

Dane, S., Borekci, B. and Kadanali, S. (2008) Right-sided lateralisation of ovarian cancer and right bias asymmetry for involved pelvic lymph nodes by ovarian cancer cells. *Laterality*, **13**, 393-402.

Dean, D., and Powers, V. (2001) Persistent *Chlamydia trachomatis* infections resist apoptotic stimuli. *Infect Immun*, **69**, 2442-2447.

Debattista, J., Gazzard, C. M., Wood, R. N., Allan, J. A. Allan, J. M, Scarman, A., Mortlock, M., Timms, P. and Knox, C. L. (2004) Interaction of microbiology and pathology in women undergoing investigations for infertility. *Infect Dis Obstet Gynecol*, **12**, 135-141.

Desai, M., ter Kuile, F. O., Nosten, F., McGready, R., Asamoa, K., Brabin, B. and Newman, R. D. (2007) Epidemiology and burden of malaria in pregnancy. *Lancet Infect Dis*, **7**, 93-104.

Devaux, A., Soula, V., Sifer, C., Branger, M., Naouri, M., Porcher, R. (2003) Hepatitis C virus detection in follicular fluid and culture media from HCV+ women, and viral risk during IVF procedures. *Hum Reprod*, ***18***, 2342-2349.

Diaz, P. I., Zilm, P. S. and Rogers, A. H. (2002) *Fusobacterium nucleatum* supports the growth of *Porphyromonas gingivalis* in oxygenated and carbon-dioxide-depleted environments. *Microbiol*, **148**, 467-472.

Dicker, D., Ashkenazai, J., Feldberg, D., Levy, T., Dekel, A. and Ben-Rafael, Z. (1993) Severe abdominal complications after trans-vaginal ultrasonographically guided retrieval of oocytes for *in vitro* fertilisation. *Hum Reprod*, **14**, 2402-2403.

Diemer, T., Weidner, W., Michelmann, H. W., Schiefer, H. G., Rovan, E. and Mayer, F. (1996) Influence of *Escherichia coli* on motility parameters of human spermatozoa *in vitro*. *Int J Androl*, **19**, 271-277.

Dikensoy, O., Filiz, A., Bayram, N., Balci, I., Zer, Y., Celik, G. and Ekinci, E. (2004) First report of pulmonary *Nocardia otitidis caviarum* infection in an immunocompetent patient from Turkey. *Int J Clin Pract*, **58**, 210-213.

Dinarello, C. A. (2000) Pro-inflammatory cytokines. *Chest*, **118**, 503-508.

Donlan, R. M. (2002) Biofilms: Microbial life on surfaces. *Emerg Infect Dis*, **8**, 881-890.

Donlan, R. M. and Costerton, J. W. (2002) Biofilms: Survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev*, **15**, 167-193.

Drew, L., and Bates, M. (1999) Cytomegalovirus. In Holmes, K. K. *et al*. (eds.) *Sexually Transmitted Diseases*. McGraw-Hill, New York.

Drobnis, E. Z. and Overstreet, J. W. (1992) Natural history of mammalian spermatozoa in the female reproductive tract. *Oxf Rev Reprod Biol*, **14**, 1-45.

Eckert, L. O., Hawes, S. E., Wolner-Hanssen, P., Money, D. M., Peeling, R. W., Brunham, R. C., Stevens, C. E., Eschenbach, D. A. and Stamm, W. E. (1997) Prevalence and correlates of antibody to chlamydial heat shock protein in women attending sexually transmitted disease clinics and women with confirmed pelvic inflammatory disease. *J Infect Dis*, **175**, 1453-1458.

Eckert, L. O., Moore, D. E., Patton, D. L., Agnew, K. J. and Eschenbach, D. A. (2003) Relationship of vaginal bacteria and inflammation with conception and early pregnancy loss following *in vitro* fertilisation. *Infect Dis Obstet Gynecol*, **11**, 11-17.

Edwards, R. G. (1974) Follicular fluid. *J Reprod Fertil*, **37**, 189-219.

Edwards, R. and Harding, K. G. (2004) Bacteria and wound healing. *Curr Opin Infect Dis*, **17**, 91-96.

Egbase, P. E., al-Sharhan, M., al-Othman, S., al-Mutawa, M., Udo, E. and Grudzinskas, J. G. (1996) Incidence of microbial growth from the tip of the embryo transfer catheter after embryo transfer in relation to clinical pregnancy rate following *in vitro* fertilisation and embryo transfer. *Hum Reprod*, **11**, 1687-1689.

Egbase, P. E., Udo, E. E., Al-Sharhan, M. and Grudzinskas, J. G. (1999) Prophylactic antibiotics and endocervical microbial inoculation of the endometrium at embryo transfer. *Lancet*, **354**, 651-652.

Eggert-Kruse, W., Gerhard, I., Naher, H., Tilgen, W. and Runnebaum, B. (1990) Chlamydial infection-a female and/or male infertility factor? *Fertil Steril*, **53**, 1037-1043.

Eggert-Kruse, W., Probst, S., Rohr, G., Aufenanger, J. and Runnebaum, B. (1995) Screening for subclinical inflammation in ejaculates. *Fertil Steril*, **64**, 1012-1022.

Eggert-Kruse, W., Botz, I., Pohl, S., Rohr, G. and Strowitzki, T. (2000) Antimicrobial activity of human cervical mucus. *HumReprod*, **15**, 778-784.

El Borai, N., LeFevre, C., Inoue, M., Naumova, E. N., Sato, K., Suzuki, S. (1998) Presence of HSV-1 DNA in semen and menstrual blood. *J Reprod Immunol, **41***, 137-147.

El-Shawarby, S., Margara, R., Trew, G. and Lavery, S. (2004) A review of complications following trans-vaginal oocyte retrieval for *in vitro* fertilisation. *Hum Fertil (Camb)*, **7**, 127-133.

El-Toukhy, T. and Hanna, L. (2006) Pelvic infection after oocyte retrieval: A preventable complication or an inevitable risk? *J Obstet Gynaecol*, **26**, 701 - 703.

Eschenbach, D. A., Buchanan, T. M., Pollock, H. M., Forsyth, P. S., Alexander, E. R., Lin, J. S., Wang, S. P., Wentworth, B. B., MacCormack, W. M. and Holmes, K. K. (1975) Polymicrobial etiology of acute pelvic inflammatory disease. *N Engl J Med*, **293**, 166-171.

Eschenbach, D. A., Gravett, M. G., Chen, K. C., Hoyme, U. B. and Holmes, K. K. (1984) Bacterial vaginosis during pregnancy. An association with prematurity and postpartum complications. *Scand J Urol Nephrol Suppl*, **86**, 213-222.

Evans, D. (1993) *Actinomyces israelii* in the female genital tract: A review. *Genitourin Med*, **69**, 54-59.

Fainaru, O., Amsalem, H., Bentov, Y., Esfandiari, N. and Casper, R. F. (2010) $CD56^{bright}$ $CD16^{-}$ natural killer cells accumulate in the ovarian follicular fluid of patients undergoing *in vitro* fertilisation. *Fertil Steril*, **94**, 1918-1921.

Fakhry, S., Manzo, N., D'Apuzzo, E., Pietrini, L., Sorrentini, I., Ricca, E., De Felice, M. and Baccigalupi, L. (2009) Characterisation of intestinal bacteria tightly bound to the human ileal epithelium. *Res Microbiol*, **160**, 817-823.

Fanchin, R., Harmas, A., Benaoudia, F., Lundkvist, U., Olivennes, F. and Frydman, R. (1998) Microbial flora of the cervix assessed at the time of embryo transfer adversely affects *in vitro* fertilisation outcome. *Fertil Steril*, **70**, 866-870.

Farooki, M. A. (1967) Epidemiology and pathology of chronic endometritis. *Int Surg*, **48**, 566-573.

Fasciani, A., D'Ambrogio, G., Bocci, G., Monti, M., Genazzani, A. R. and Artini, P. G. (2000) High concentrations of the vascular endothelial growth factor and interleukin-8 in ovarian endometriomata. *Mol Hum Reprod*, **6**, 50-54.

Feghali, J., Bakar, J., Mayenga, J. M., Segard, L., Hamou, J., Driguez, P. and Belaisch-Allart, J. (2003) [Systematic hysteroscopy prior to *in vitro* fertilisation]. *Gynecol Obstet Fertil*, **31**, 127-131. Abstract only

Fey, P. D. (2010) Modality of bacterial growth presents unique targets: How do we treat biofilm-mediated infections? *Curr Opin Microbiol*, **13**, 610-615.

Fey, P. D. and Olson, M. E. (2010) Current concepts in biofilm formation of *Staphylococcus epidermidis*. *Future Microbiol*, **5**, 917-933.

Fidel, P. L., Jr., Cutright, J. L., Tait, L. and Sobel, J. D. (1996) A murine model of *Candida glabrata* vaginitis. *J Infect Dis*, **173**, 425-431.

Fidel, P. L., Jr., Ginsburg, K. A., Cutright, J. L., Wolf, N. A., Leaman, D., Dunlap, K. and Sobel, J. D. (1997) Vaginal-associated immunity in women with recurrent vulvovaginal candidiasis: Evidence for vaginal Th1-type responses following intravaginal challenge with candida antigen. *J Infect Dis*, **176**, 728-739.

Figura, N., Piomboni, P., Ponzetto, A., Gambera, L., Lenzi, C., Vaira, D., Peris, C., Lotano, M., Gennari, L., Bianciardi, L., Renieri, T., Valensin, P., Capitani, S., Moretti, E., Colapinto, R., Baccetti, B., Gennari, C. (2002) *Helicobacter pylori* infection and infertility. *Eu J Gastroenterol*, **14**, 663-669.

Fiorino, A. (1996) Intrauterine contraceptive device-associated actinomycotic abscess and actinomyces detection on cervical smear. *Obstet Gynecol*, **87**, 142-149.

Fisher, M. E., Nisenbaum, H. L., Axel, L. and Broderick, G. A. (1998) Prostatic abscess due to *Aspergillus fumigatus*: TRUS and MR imaging findings. *J Ultrasound Med*, **17**, 181-184.

Flemming, H. C. and Wingender, J. (2001) Relevance of microbial extracellular polymeric substances (EPSS) part II: Technical aspects. *Water Sci Technol*, **43**, 9-16.

Fontaine, E. A., Bryant, T. N., Taylor-Robinson, D., Borriello, S. P. and Davies, H. A. (1986) A numerical taxonomic study of anaerobic Gram-negative bacilli classified as *Bacteroides ureolyticus* isolated from patients with non-gonococcal urethritis. *J Gen Microbiol*, **132**, 3137-3146.

Fowler, A. V. and Zabin, I. (1977) The amino acid sequence of β-galactosidase of *Escherichia coli*. *Proc Natl Acad Sci U S A*, **74**, 1507-1510.

Fowlkes, D. M., Dooher, G. B. and O'Leary, W. M. (1975) Evidence by scanning electron microscopy for an association between spermatozoa and T-mycoplasmas in men of infertile marriage. *Fertil Steril*, **26**, 1203-1211.

Friberg, J., Confino, E., Suarez, M. and Gleicher, N. (1987) *Chlamydia trachomatis* attached to spermatozoa recovered from the peritoneal cavity of patients with salpingitis. *J Reprod Med*, **32**, 120-122.

Friedman, G., Jankowski, S., Marchant, A., Goldman, M., Kahn, R. J. and Vincent, J. L. (1997) Blood interleukin-10 levels parallel the severity of septic shock. *J Crit Care*, **12**, 183-187.

Fukuda, M., Fukuda, K., Andersen, C. Y. and Byskov, A. G. (2000) Right-sided ovulation favours pregnancy more than left-sided ovulation. *Hum Reprod*, **15**, 1921-1926.

Furr, P. M. and Taylor-Robinson, D. (1993) The contrasting effects of progesterone and oestrogen on the susceptibility of mice to genital infection with *Mycoplasma pulmonis*. *J Med Microbiol*, **38**, 160-165.

Furr, P. M. and Taylor-Robinson, D. (1991) The influence of hormones on the bacterial flora of the murine vagina and implications for human disease. *Microbiol Ecol Health dis*, **4**, 141-148.

Furukawa, K., Lieu, P. K., Tokitoh, H. and Fujii, T. (2006) Development of single-stage nitrogen removal using anammox and partial nitritation (snap) and its treatment performances. *Water Sci Technol*, **53**, 83-90.

Fux, C. A., Costerton, J. W., Stewart, P. S. and Stoodley, P. (2005 a) Survival strategies of infectious biofilms. *Trends Microbiol*, **13**, 34-40.

Fux, C. A., Shirtliff, M., Stoodley, P. and Costerton, J. W. (2005 b) Can laboratory reference strains mirror "real-world" pathogenesis? *Trends Microbiol*, **13**, 58-63.

Gagliardi, M. C., Starnino, S., Teloni, R., Mariotti, S., Dal Conte, I., Di Carlo, A. and Stefanelli, P. (2011) Circulating levels of interleukin-17A and interleukin-23 are increased in patients with gonococcal infection. *FEMS Immunol Med Microbiol*, **61**,129-32.

Gallegos, G., Ramos, B., Santiso, R., Goyanes, V., Gosalvez, J. and Fernandez, J. L. (2008) Sperm DNA fragmentation in infertile men with genitourinary infection by *Chlamydia trachomatis* and mycoplasma. *Fertil Steril*, **90**, 328-334.

Gardner, D. K., Schoolcraft, W. B., Wagley, L., Schlenker, T., Stevens, J. and Hesla, J. (1998) A prospective randomized trial of blastocyst culture and transfer in *in vitro* fertilisation. *Hum Reprod*, **13**, 3434-3440.

Gaudoin, M., Rekha, P., Morris, A., Lynch, J. and Acharya, U. (1999) Bacterial vaginosis and past chlamydial infection are strongly and independently associated with tubal infertility but do not affect *in vitro* fertilisation success rates. *Fertil Steril*, **72**, 730-732.

Gazvani, M. R., Christmas, S., Quenby, S., Kirwan, J., Johnson, P. M. and Kingsland, C. R. (1998) Peritoneal fluid concentrations of interleukin-8 in women with endometriosis: Relationship to stage of disease. *Hum Reprod*, **13**, 1957-1961.

Gazvani, M. R., Bates, M., Vince, G., Christmas, S., Lewis-Jones, D. I. and Kingsland, C. (2000) Follicular fluid concentrations of interleukin-12 and interleukin-8 in IVF cycles. *Fertil Steril*, **74**, 953-958.

Ge, D. and You, Z. (2008) Expression of interleukin-17rc protein in normal human tissues. *Int Arch Med*, **1**, 19.

Geary, S. and Moon, Y. (2006) The human embryo *in vitro*. *J Reprod Med*, **51**, 293-302.

Geisler, W. M. and Krieger, J. N. (2008) Epididymitis. In Holmes, K. K. *et al*., (eds.) *Sexually Transmitted Diseases*. McGraw-Hill, New York.

Gelfand, M., Ross, M., Blair, D., Weber, M. (1971) Distribution and extent of schistosomiasis in female pelvic organs, with special reference to the genital tract, as determined at autopsy. *Am J Trop Med and Hyg*, **20**, 846-849.

Gerber, S., Vardhana, S., Meagher-Villemure, K., Vial, Y., Hohlfeld, P. and Witkin, S. S. (2005) Association between fetal interleukin-1 receptor antagonist gene polymorphism and unexplained fetal death. *A J Obstet Gynecol*, **193**, 1472-1477.

Gerendai, I. and Halasz, B. (1997) Neuroendocrine asymmetry. *Front Neuroendocrinol*, **18**, 354-381.

Geva, E., Lessing, J. B., Lerner-Geva, L., Azem, F., Yovel, I., Ben-Yosef, D., Barkai, U. and Amit, A. (1997) Interleukin-10 in preovulatory follicular fluid of patients undergoing *in vitro* fertilisation and embryo transfer. *Am J Reprod Immunol*, **37**, 187-190.

Gibbs, R. S., Romero, R., Hillier, S. L., Eschenbach, D. A. and Sweet, R. L. (1992) A review of premature birth and subclinical infection. *Am J Obstet Gynecol*, **166**, 1515-1528.

Ginsburg, E. S., Xiao, L., Gargiulo, A. R., Kung, F. T., Politch, J. A., Schust, D. J. and Hill, J. A. (2005) T-helper 2 and 3 type immunity to trophoblast in successful *in vitro* fertilisation-embryo transfer. *Fertil Steril*, **83**, 1659-1664.

Giridhar, G., Kreger, A. S., Myrvik, Q. N. and Gristina, A. G. (1994) Inhibition of staphylococcus adherence to biomaterials by extracellular slime of *S. epidermidis* rp12. *J Biomed Mater Res*, **28**, 1289-1294.

Goldenberg, R. L., Hauth, J. C. and Andrews, W. W. (2000) Intrauterine infection and preterm delivery. *N Engl J Med*, **342**, 1500-1507.

Gomez, C. I., Stenback, W. A., James, A. N., Criswell, B. S. and Williams, R. P. (1979) Attachment of *Neisseria gonorrhoeae* to human sperm. Microscopical study of trypsin and iron. *Br J Vener Dis*, **55**, 245-255.

Gordon, C. J. and Rowsey, P. J. (1999) Are circulating cytokines interleukin-6 and tumor necrosis factor α involved in chlorpyrifos-induced fever? *Toxicology*, **134**, 9-17.

Gorga, F., Galdiero, M., Buommino, E. and Galdiero, E. (2001) Porins and lipopolysaccharide induce apoptosis in human spermatozoa. *Clin Diagn Lab Immunol*, **8**, 206-208.

Gould, D. (2010) Causes, prevention and treatment of *Escherichia coli* infections. *Nurs Stand*, **24**, 50-56.

Goulet, M., Dular, R., Tully, J. G., Billowes, G. and Kasatiya, S. (1995 b) Isolation of *Mycoplasma pneumoniae* from the human urogenital tract. *J Clin Microbiol*, **33**, 2823-2825.

Goverde, A. J., Schats, R., van Verlo, P.J.M. and Claessen, F.A.P. (1996) An unexpected guest in follicular fluid. *Hum Reprod*, **11**, 531-532.

Gravett, M. G., Hummel, D., Eschenbach, D. A. and Holmes, K. K. (1986) Preterm labor associated with subclinical amniotic fluid infection and with bacterial vaginosis. *Obstet Gynecol*, **67**, 229-237.

Gravett, M. G., Hitti, J., Hess, D. L. and Eschenbach, D. A. (2000) Intrauterine infection and preterm delivery: Evidence for activation of the fetal hypothalamic-pituitary-adrenal axis. *Am J Obstet Gynecol*, **182**, 1404-1413.

Greisen, K., Loeffelholz, M., Purohit, A. and Leong, D. (1994) PCR primers and probes for the 16S rRNA gene of most species of pathogenic bacteria, including bacteria found in cerebrospinal fluid. *J Clin Microbiol*, **32**, 335-351.

Guillet-Rosso, F., Fari, A., Taylor, S., Forman, R., Belaisch-Allart, J., Testart, J. and Frydman, R. (1987) Systematic semen culture and its influence on IVF management. *Br J Obstet Gynaecol*, **94**, 543-547.

Gurgan, T., Urman, B., Diker, K. S., Delilbasi, L. and Kisnisci, H. A. (1993) Human follicular fluid from pre-ovulatory follicles in patients undergoing *in vitro* fertilisation inhibits the *in vitro* growth of Gram-positive microorganisms. *Hum Reprod*, **8**, 508-510.

Halling, S. M., Jensen, A. E. and Olsen, S. C. (2008) Defensin susceptibility and colonisation in the mouse model of aj100, a polymyxin b-resistant, *Brucella abortus* rb51 isolate. *Curr Microbiol*, **56**, 274-278.

Hammadeh, M. E., Fischer-Hammadeh, C., Georg, T., Rosenbaum, P. and Schmidt, W. (2003) Comparison between cytokine concentration in follicular fluid of poor and high responder patients and their influence of ICSI-outcome. *Am J Reprod Immunol*, **50**, 131-136.

Hammadeh, M. E., Fischer-Hammadeh, C., Hoffmeister, H., Herrmann, W., Rosenbaum, P. and Schmidt, W. (2004) Relationship between cytokine concentrations (FGF, sICAM-1 and SCF) in serum, follicular fluid and ICSI outcome. *Am J Reprod Immunol*, **51**, 81-85.

Han, Y. W. (2011) Oral health and adverse pregnancy outcomes - what's next? *J Dent Res*, **90**, 289-293.

Handsfield, H. H. (1983) Recent developments in gonorrhoea and pelvic inflammatory disease. *J Med*, **14**, 281-305.

Handyside, A. H., Lesko, J. G., Tarin, J. J., Winston, R. M. and Hughes, M. R. (1992) Birth of a normal girl after *in vitro* fertilisation and preimplantation diagnostic testing for cystic fibrosis. *N Engl J Med*, **327**, 905-909.

Harro, J. M., Peters, B. M., O'May, G. A., Archer, N., Kerns, P., Prabhakara, R. and Shirtliff, M. E. (2010) Vaccine development in *Staphylococcus aureus*: Taking the biofilm phenotype into consideration. *FEMS Immunol Med Microbiol*, **59**, 306-323.

Hartmann, K. A., Lerand, S. J. and Jay, M. S. (2009) Tubo-ovarian abscess in virginal adolescents: Exposure of the underlying etiology. *J Pediatr Adolesc Gynecol*, **22**, e13-16.

Hauth, J. C. and Andrews, W. W. (1998) Use of antibiotics to prevent preterm birth. *Am J Obstet Gynecol*, **178**, 624-625.

Hay, P. (2000) Recurrent bacterial vaginosis. *Curr Infect Dis Rep*, **2**, 506-512.

Hay, P. E., Taylor-Robinson, D. and Lamont, R. F. (1992) Diagnosis of bacterial vaginosis in a gynaecology clinic. *Br J Obstet Gynaecol*, **99**, 63-66.

Heinonen, P. K. and Miettinen, A. (1994) Laparoscopic study on the microbiology and severity of acute pelvic inflammatory disease. *Eu J Obstet Gynecol and Reprod Biol*, **57**, 85-89.

Henkel, R., Hajimohammad, M., Stalf, T., Hoogendijk, C., Mehnert, C., Menkveld, R., Gips, H., Schill, W. B. and Kruger, T. F. (2004) Influence of deoxyribonucleic acid damage on fertilisation and pregnancy. *Fertil Steril*, **81**, 965-972.

Henry-Suchet, J., Catalan, F., Loffredo, V., Serfaty, D., Siboulet, A., Perol, Y., Sanson, M. J., Debache, C., Pigeau, F., Coppin, R., de Brux, J. and Poynard, T. (1980) Microbiology of specimens obtained by laparoscopy from controls and from patients with pelvic inflammatory disease or infertility with tubal obstruction: *Chlamydia trachomatis* and *Ureaplasma urealyticum*. *Am J Obstet Gynecol*, **138**, 1022-1025.

Hill, A. C., Tucker, M. J., Whittingham, D. G. and Craft, I. (1987) Mycoplasmas and *in vitro* fertilisation. *Fertil Steril*, **47**, 652-655.

Hill, G.A., and Osteen, K.G. (1989) Follicular fluid steroid content and *in vitro* steroid secretion by granulosa-lutein cells from individual follicles among different stimulation protocols for *in vitro* fertilisation-embryo transfer. *J In vitro Fert Embryo Transf*, **6**, 201-206.

Hill, G. B. (1993) Investigating the source of amniotic fluid isolates of fusobacteria. *Clin Infect Dis*, **16**, S423-424.

Hill, J. A., Welch, W. R., Faris, H. M. and Anderson, D. J. (1990) Induction of class II major histocompatibility complex antigen expression in human granulosa cells by interferon γ: A potential mechanism contributing to autoimmune ovarian failure. *Am J Obstet Gynecol*, **162**, 534-540.

Hillier, S. L., Marrazzo, J. M. and Holmes, K. K. (2008) Bacterial Vaginosis. In Holmes, K. K.. *et al*. (eds.) *Sexually Transmitted Diseases*. McGraw-Hill, New York.

Hillier, S., Krohn, M. A., Watts, D. H., Wolner-Hanssen, P., Eschenbach, D., Hillier, S. L., Rabe, L. K., Klebanoff, S. J. and Eschenbach, D. A. (1990) Microbiologic efficacy of intravaginal clindamycin cream for the treatment of bacterial vaginosis. *Obstet Gynecol*, **76**, 407-413.

Hillier, S., Witkin, S., Krohn, M., Watts, D., Kiviat, N. and Eschenbach, D. (1993) The relationship of amniotic fluid cytokines and preterm delivery, amniotic fluid infection, histologic chorioamnionitis, and chorioamnion infection. *Obstet Gynecol*, **81**, 941-948.

Hillier, S. L., Martius, J., Krohn, M., Kiviat, N., Holmes, K. K. and Eschenbach, D. A. (1988) A case-control study of chorioamnionic infection and histologic chorioamnionitis in prematurity. *N Engl J Med*, **319**, 972-978.

Hillier, S. L., Nugent, R. P., Eschenbach, D. A., Krohn, M. A., Gibbs, R. S., Martin, D. H., Cotch, M. F., Edelman, R., Pastorek, J. G., 2nd, Rao, A. V. and *et al*. (1995) Association between bacterial vaginosis and preterm delivery of a low-birth-weight infant. The vaginal infections and prematurity study group. *N Engl J Med*, **333**, 1737-1742.

Hillier, S. L., Kiviat, N. B., Hawes, S. E., Hasselquist, M. B., Hanssen, P. W., Eschenbach, D. A. and Holmes, K. K. (1996) Role of bacterial vaginosis-associated microorganisms in endometritis. *Am J Obstet Gynecol*, **175**, 435-441.

Hillier, S. L. (2008) Normal genital flora. In Holmes, K. K. *et al*. (eds.) *Sexually Transmitted Diseases*. McGraw-Hill, New York.

Hirota, Y., Osuga, Y., Yoshino, O., Koga, K., Yano, T., Hirata, T., Nose, E., Ayabe, T., Namba, A., Tsutsumi, O. And Taketani, Y. (2003) Possible roles of thrombin-induced activation of protease-activated receptor 1 in human luteinized granulosa cells. *J Clin Endocrinol Metab*, **88**, 3952-3957.

Ho, H. N., Wu, M. Y., Chao, K. H., Chen, C. D., Chen, S. U., Chen, H. F. and Yang, Y. S. (1996) Decrease in interferon γ production and impairment of T-lymphocyte proliferation in peritoneal fluid of women with endometriosis. *Am J Obstet Gynecol*, **175**, 1236-1241.

Ho, H. N., Wu, M. Y., Chao, K. H., Chen, C. D., Chen, S. U. and Yang, Y. S. (1997) Peritoneal interleukin-10 increases with decrease in activated CD4+ T lymphocytes in women with endometriosis. *Hum Reprod*, **12**, 2528-2533.

Holmes, K. K., Sparling, P. F., Mardh, P., Lemon, S. T., Stamm, S. E., Piot, P., Wasserheit, J. N (2008) *Sexually Transmitted Diseases*. McGraw-Hill, New York.

Holst, E., Goffeng, A. R. and Andersch, B. (1994) Bacterial vaginosis and vaginal microorganisms in idiopathic premature labor and association with pregnancy outcome. *J Clin Microbiol*, **32**, 176-186.

Hook, C. E., Matyszak, M. K. and Gaston, J. S. (2005) Infection of epithelial and dendritic cells by *Chlamydia trachomatis* results in IL-18 and IL-12 production, leading to interferon γ production by human natural killer cells. *FEMS Immunol Med Microbiol*, **45**, 113-120.

Hook, E. I. and Handsfield, H. (1999) Gonococcal infections in the adult. In Holmes, K. K. *et al.* (eds.) *Sexually Transmitted Diseases*. McGraw-Hill, New York.

Horne, A. W., Stock, S. J. and King, A. E. (2008) Innate immunity and disorders of the female reproductive tract. *Reproduction*, **135**, 739-749.

Hosseinzadeh, S., Brewis, I. A., Eley, A. and Pacey, A. A. (2001) Co-incubation of human spermatozoa with *Chlamydia trachomatis* serovar E causes premature sperm death. *Hum Reprod*, **16**, 293-299.

Howard, T. L. (1971) Bacterial hitch-hikers. *J Urol*, **106**, 94.

Hu, S. K., Mitcho, Y. L. and Rath, N. C. (1988) Effect of oestradiol on interleukin-1 synthesis by macrophages. *Int J Immunopharmacol*, **10**, 247-252.

Husband, A. J., Bao, S. and Beagley, K. W. (1999) Analysis of the mucosal microenvironment: Factors determining successful responses to mucosal vaccines. *Vet Immunol Immunopathol*, **72**, 135-142.

Huyser, C., Fourie, F. L., Oosthuizen, M. and Neethling, A. (1991) Microbial flora in semen during *in vitro* fertilisation. *J In vitro Fert Embryo Transf*, **8**, 260-264.

Hvid, M., Baczynska, A., Deleuran, B., Fedder, J., Knudsen, H. J., Christiansen, G. and Birkelund, S. (2007) Interleukin-1 is the initiator of Fallopian tube destruction during *Chlamydia trachomatis* infection. *Cell Microbiol*, **9**, 2795-2803.

Ingalls, R., Rice, P., Qureshi, N., Takayama, K., Lin, J. and Golenbock, D. (1995) The inflammatory cytokine response to *Chlamydia trachomatis* infection is endotoxin mediated. *Infect Immun*, **63**, 3125-3130.

Isenberg, H. D. (ed) (2009) Clinical Microbiology Procedures Handbook. American Society for Microbiology, Washington.

Jacobsson, B., Pernevi, P., Chidekel, L. and Jorgen Platz-Christensen, J. (2002) Bacterial vaginosis in early pregnancy may predispose for preterm birth and postpartum endometritis. *Acta Obstet Gynecol Scand*, **81**, 1006-1010.

Jakobsson, T. and Forsum, U. (2008) Changes in the predominant human lactobacillus flora during *in vitro* fertilisation. *Ann Clin Microbiol Antimicrob*, **7**, 14-22.

Jakubovics, N. S. (2010) Talk of the town: Interspecies communication in oral biofilms. *Mol Oral Microbiol*, **25**, 4-14.

Jakubovics, N. S. and Kolenbrander, P. E. (2010) The road to ruin: The formation of disease-associated oral biofilms. *Oral Dis*, **16**, 729-739.

James-Holmquest, A. N., Swanson, J., Buchanan, T. M., Wende, R. D. and Williams, R. P. (1974) Differential attachment by piliated and nonpiliated *Neisseria gonorrhoeae* to human sperm. *Infect Immun*, **9**, 897-902.

Jasper, M. and Norman, R. J. (1995) Endocrinology: Immunoactive interleukin-1 β and tumour necrosis factor-α in thecal, stromal and granulosa cell cultures from normal and polycystic ovaries. *Hum Reprod*, **10**, 1352-1354.

Jensen, J. S., Borre, M. B. and Dohn, B. (2003) Detection of *Mycoplasma genitalium* by PCR amplification of the 16S rRNA gene. *J Clin Microbiol*, **41**, 261-266.

Jha, R., Srivastava, P., Salhan, S., Finckh, A., Gabay, C., Mittal, A. and Bas, S. (2011) Spontaneous secretion of interleukin-17 and -22 by human cervical cells in *Chlamydia trachomatis* infection. *Microbes Infect*, **13**, 167-178.

Kanakas, N., Mantzavinos, T., Boufidou, F., Koumentakou, I. and Creatsas, G. (1999) *Ureaplasma urealyticum* in semen: Is there any effect on *in vitro* fertilisation outcome? *Fertil Steril*, **71**, 523-527.

Kapranos, N., Petrakou, E., Anastasiadou, C., & Kotronias, D. (2003) Detection of herpes simplex virus, cytomegalovirus, and Epstein-Barr virus in the semen of men attending an infertility clinic. *Fertil Steril*, ***79 Suppl 3***, 1566-1570.

Karagouni, E. E., Chryssikopoulos, A., Mantzavinos, T., Kanakas, N. and Dotsika, E. N. (1998) Interleukin-1 β and interleukin-1 α may affect the implantation rate of patients undergoing *in vitro* fertilisation-embryo transfer. *Fertil Steril*, **70**, 553-559.

Katz, E., Goodman, A. L., Garcia, J. and Rone, J. D. (1992) Evidence for the production of a nonsteroidal endotheliotropic factor by human granulosa cells in culture. *Fertil Steril*, **57**, 107-112.

Kaul, R., Rebbapragada, A., Hirbod, T., Wachihi, C., Ball, T.B., Plummer, F.A., Kimani, J. and Jaoko, W. (2008) Genital levels of soluble immune factors with anti-HIV activity may correlate with increased HIV susceptibility. *AIDS*, **22**, 2049-2051.

Kawano, K., Kato, Y. and Tsunoda, Y. (2004) Comparison of *in vitro* development of porcine nuclear-transferred oocytes receiving fetal somatic cells by injection and fusion methods. *Cloning Stem Cells*, **6**, 67-72.

Keith, L. G., Berger, G. S., Edelman, D. A., Newton, W., Fullan, N., Bailey, R. and Friberg, J. (1984) On the causation of pelvic inflammatory disease. *Am J Obstet Gynecol*, **149**, 215-224.

Keller, M. and Ramos, J. L. (2008) Microbial goods from single cells and metagenomes. *Curr Opin Microbiol*, **11**, 195-197.

Kepkep, K., Tuncay, Y. A. and Yigitbasi, R. (2006) Nocardial tubo-ovarian abscess in a pregnant woman: A rare case report. *Aust N Z J Obstet Gynaecol*, **46**, 363-365.

Kilic, S., Evsen, M., Tasdemir, N., Yilmaz, N., Yuksel, B., Dogan, M. and Batioglu, S. (2007) Follicular fluid vascular endothelial growth factor and tumour necrosis factor α concentrations in patients with endometriosis undergoing ICSI. *Reprod Biomed Online*, **15**, 316-320.

Kilic, S., Moraloglu, O., Yuksel, B., Ozaksit, G., Tasdemir, N. and Dogan M. (2009) IL-18 and IL-18 binding protein concentrations in serum and ovarian follicular fluid do not affect *in vitro* fertilisation outcomes in obese, overweight, and non-obese patients. *J Reprod Immunol*. **79**, 211-214

Kim, S. W., Nah, M. Y., Yeum, C. H., Kim, N. H., Choi, H. S., Juhng, S. W., Choi, K. C., Toy, E. C., Scerpella, E. G., Riggs, J. W., Kostelnik, F. V., Fremount, H. N., Saw, E. C., Smale, L. E., Einstein, H., Huntington, R. W., Jr., Craig, M. W., Davey, W. N., Green, R. A. and Plaut, A. (2001) Pelvic aspergillosis with tubo-ovarian abscess in a renal transplant recipient. *J Infect*, **42**, 215-217.

King, C. (2008) T helper cell differentiation: IL-21 and T helper cell differentiation: Jack of all trades? *Immunol Cell Biol*, **86**, 554-556.

King, J. A., Olsen, T. G., Lim, R. and Nycum, L. R. (2002) *Pseudomonas aeruginosa*-infected IUD associated with pelvic inflammatory disease. A case report. *J Reprod Med*, **47**, 1035-1037.

Kiviat, N. B., Paavonen, J. A., Wolner-Hanssen, P., Critchlow, C. W., Stamm, W. E., Douglas, J., Eschenbach, D. A., Corey, L. A. and Holmes, K. K. (1990 a) Histopathology of endocervical infection caused by *Chlamydia trachomatis*, herpes simplex virus, *Trichomonas vaginalis*, and *Neisseria gonorrhoeae*. *Hum Pathol*, **21**, 831-837.

Kiviat, N. B., Wolner-Hanssen, P., Eschenbach, D. A., Wasserheit, J. N., Paavonen, J. A., Bell, T. A., Critchlow, C. W., Stamm, W. E., Moore, D. E. and Holmes, K. K. (1990 b) Endometrial histopathology in patients with culture-proved upper genital tract infection and laparoscopically diagnosed acute salpingitis. *Am J Surg Pathol*, **14**, 167-175.

Klausen, M., Aaes-Jorgensen, A., Molin, S. and Tolker-Nielsen, T. (2003 a) Involvement of bacterial migration in the development of complex multicellular structures in *Pseudomonas aeruginosa* biofilms. *Mol Microbiol*, **50**, 61-68.

Klausen, M., Heydorn, A., Ragas, P., Lambertsen, L., Aaes-Jorgensen, A., Molin, S. and Tolker-Nielsen, T. (2003 b) Biofilm formation by *Pseudomonas aeruginosa* wild type, flagella and type iv pili mutants. *Mol Microbiol*, **48**, 1511-1524.

Klebanoff, S. J. and Coombs, R. W. (1991) Viricidal effect of *Lactobacillus acidophilus* on human immunodeficiency virus type 1: Possible role in heterosexual transmission. *J Exp Med*, **174**, 289-292.

Klebanoff, S. J., Hillier, S. L., Eschenbach, D. A. and Waltersdorph, A. M. (1991) Control of the microbial flora of the vagina by H_2O_2-generating lactobacilli. *J Infect Dis*, **164**, 94-100.

Klein, C. M. and Burden, H. W. (1988) Anatomical localisation of afferent and postganglionic sympathetic neurons innervating the rat ovary. *Neurosci Lett*, **85**, 217-222.

Knox, C. L. and Timms, P. (1998) Comparison of PCR, nested PCR, and random amplified polymorphic DNA PCR for detection and typing of *Ureaplasma urealyticum* in specimens from pregnant women. *J Clin Microbiol*, **36**, 3032-3039.

Knox, C. L., Allan, J. A., Allan, J. M., Edirisinghe, W. R., Stenzel, D., Lawrence, F. A., Purdie, D. M. and Timms, P. (2003) *Ureaplasma parvum* and *Ureaplasma urealyticum* are detected in semen after washing before assisted reproductive technology procedures. *Fertil Steril*, **80**, 921-929.

Knudsen, A., Benfield, T. and Kofoed, K. (2009) Cytokine expression during syphilis infection in HIV-1-infected individuals. *Sex Transm Dis*. **36**, 300-304.

Koch, A. E., Polverini, P. J., Kunkel, S. L., Harlow, L. A., DiPietro, L. A., Elner, V. M., Elner, S. G. and Strieter, R. M. (1992) Interleukin-8 as a macrophage-derived mediator of angiogenesis. *Science*, **258**, 1798-1801.

Kolenbrander, P. E., Palmer, R. J., Jr., Rickard, A. H., Jakubovics, N. S., Chalmers, N. I. and Diaz, P. I. (2006) Bacterial interactions and successions during plaque development. *Periodontol 2000*, **42**, 47-79.

Korn, A. P., Bolan, G., Padian, N., Ohm-Smith, M., Schachter, J. and Landers, D. V. (1995) Plasma cell endometritis in women with symptomatic bacterial vaginosis. *Obstet Gynecol*, **85**, 387-390.

Kornman, K. S. and Loesche, W. J. (1982) Effects of oestradiol and progesterone on *Bacteroides melaninogenicus* and *Bacteroides gingivalis*. *Infect Immun*, **35**, 256-263.

Koskela, P., Antilla, T., Bjorge, T (2000) *Chlamydia trachomatis* infection as a risk for cervical squamous cell carcinoma. *Int J Cancer*, **85**, 35-39.

Kostelnik, F. V., Fremount, H. N., Saw, E. C., Smale, L. E., Einstein, H., Huntington, R. W., Jr., Craig, M. W., Davey, W. N., Green, R. A. and Plaut, A. (1976) Mycotic tubo-ovarian abscess associated with the intrauterine device. *Am J Obstet Gynecol*, **125**, 272-274.

Kreth, J., Vu, H., Zhang, Y. and Herzberg, M. C. (2009) Characterisation of hydrogen peroxide-induced DNA release by *Streptococcus sanguinis* and *Streptococcus gordonii*. *J Bacteriol*, **191**, 6281-6291.

Krieger, J. N. 92008) Prostatitis Syndromes: Causes, differential Diagnosis, and Clinical Management. In Holmes, K. K. *et al*. (eds.) *Sexually Transmitted Diseases*. McGraw-Hill, New York.

Krizan, J., Cuchalova, L., Sima, P., Kralickova, M., Madar, J. and Vetvicka, V. (2009) Altered distribution of NK and NKT cells in follicular fluid is associated with IVF outcome. *J Reprod Immunol*, **82**, 84-88.

Krohn, M. A., Hillier, S. L., Nugent, R. P., Cotch, M. F., Carey, J. C., Gibbs, R. S. and Eschenbach, D. A. (1995) The genital flora of women with intraamniotic infection. Vaginal infection and prematurity study group. *J Infect Dis*, **171**, 1475-1480.

Kurotsuchi, S., Ando, H., Iwase, A., Ishida, Y., Hamajima, N. and Kikkawa, F. (2008) The plausibility of *Helicobacter pylori*-related infertility in Japan. *Fertil Steril*, **90**, 866-868.

Kushnir, M. M., Naessen, T., Kirilovas, D., Chaika, A., Nosenko, J., Mogilevkina, I., Rockwood, A. L., Carlstrom, K. and Bergquist, J. (2009) Steroid profiles in ovarian follicular fluid from regularly menstruating women and women after ovarian stimulation. *Clin Chem*, **55**, 519-526.

Laato, M., Niinikoski, J., Lundberg, C. and Gerdin, B. (1988) Inflammatory reaction and blood flow in experimental wounds inoculated with *Staphylococcus aureus*. *Eur Surg Res*, **20**, 33-38.

Labbe, A. C., Frost, E., Deslandes, S., Mendonca, A. P., Alves, A. C. and Pepin, J. (2002) *Mycoplasma genitalium* is not associated with adverse outcomes of pregnancy in Guinea-Bissau. *Sex Transm Infect*, **78**, 289-291.

Laird, S. M., Tuckerman, E. M. and Li, T. C. (2006) Cytokine expression in the endometrium of women with implantation failure and recurrent miscarriage. *Reprod Biomed Online*, **13**, 13-23.

Lan, K. C., Huang, F. J., Lin, Y. C., Kung, F. T., Lan, T. H. and Chang, S. Y. (2010) Significantly superior response in the right ovary compared with the left ovary after stimulation with follicle-stimulating hormone in a pituitary down-regulation regimen. *Fertil Steril*, **93**, 2269-2273.

Landers, D. V. and Sweet, R. L. (1983) Tubo-ovarian abscess: Contemporary approach to management. *Rev Infect Dis*, **5**, 876-884.

Landgren, B. M., Ljung-Wadstrom, A., Wikborn, C., Eneroth, P. and Nord, C. E. (1984) Microbial findings in genital secretions from seven healthy fertile couples. *Med Microbiol Immunol*, **173**, 179-185.

Landis, S. J. (2008) Chronic wound infection and antimicrobial use. *Adv Skin Wound Care*, **21**, 531-540.

Last, R. G. (1984) *Anatomy: Regional and applied*. 7 th edn, Churchill Livingstone, Edinburgh.

Law, H., Itkonnen, O., Lingwood, C. (1988) A sulfogalactolipid binding protein slip 1: A conserved function for a conserved protein. *J Cell Physiol*, **137**, 462-468.

Lédée, N., Petitbarat, M., Rahmati, M., Dubanchet, S., Chaouat, G., Sandra, O., Perrier-d'Hauterive, S., Munaut, C. and Foidart, J. M. (2011) New pre-conception immune biomarkers for clinical practice: interleukin-18, interleukin-15 and TWEAK on the endometrial side, GCSF on the follicular side. *J Reprod Immunol*, **88**, 118-23.

Lefevre, J. C., Averous, S., Bauriaud, R., Blanc, C., Bertrand, M. A. and Lareng, M. B. (1988) Lower genital tract infections in women: Comparison of clinical and epidemiologic findings with microbiology. *Sex Transm Dis*, **15**, 110-113.

Li, H., Xu, L., Wang, J., Wen, Y., Vuong, C., Otto, M. and Gao, Q. (2005) Conversion of *Staphylococcus epidermidis* strains from commensal to invasive by expression of the ica locus encoding production of biofilm exopolysaccharide. *Infect Immun*, **73**, 3188-3191.

Lim, K. J., Odukoya, O. A., Ajjan, R. A., Li, T. C., Weetman, A. P. and Cooke, I. D. (2000) The role of T-helper cytokines in human reproduction. *Fertil Steril*, **73**, 136-142.

Lingwood, C., Schramayr, S. and Quinn, P. (1990 a) Male germ cell specific sulfogalactoglycerolipid is recognized and degraded by mycoplasmas associated with male infertility. *J Cell Physiol*, **142**, 170-176.

Lingwood, C. A., Quinn, P. A., Wilansky, S., Nutikka, A., Ruhnke, H. L. and Miller, R. B. (1990 b) Common sulfoglycolipid receptor for mycoplasmas involved in animal and human infertility. *Biol Reprod*, **43**, 694-697.

Linjawi, S., Li, T. C., Tuckerman, E. M., Blakemore, A. I. and Laird, S. M. (2004) Expression of interleukin-11 receptor α and interleukin-11 protein in the endometrium of normal fertile women and women with recurrent miscarriage. *J Reprod Immunol*, **64**, 145-155.

Liu, S., Wang, S., Wu, Y., Zhao, F., Zeng, T., Zhang, Y., Zhang, Q. and Gao, D. (2010) Production of pro-inflammatory cytokines in the human THP-1 monocyte cell line following induction by Tp0751, a recombinant protein of *Treponema pallidum*. *Sci China Life Sci*. **53**, 229-33.

Liversedge, N. H., Jenkins, J. M., Keay, S. D., McLaughlin, E. A., Al-Sufyan, H., Maile, L. A., Joels, L. A. and Hull, M. G. R. (1996) Antibiotic treatment based on seminal cultures from asymptomatic male partners in *in vitro* fertilisation is unnecessary and may be detrimental. *Hum Reprod*, **11**, 1227-1231.

Liversedge, N. H., Turner, A., Horner, P. J., Keay, S. D., Jenkins, J. M. and Hull, M. G. (1999) The influence of bacterial vaginosis on *in vitro* fertilisation and embryo implantation during assisted reproduction treatment. *Hum Reprod*, **14**, 2411-2415.

Lopez, D., Vlamakis, H. and Kolter, R. (2010) Biofilms. *Cold Spring Harb Perspect Biol*, **2**, 398-409.

Loret de Mola, G. J., Hecht, B.R., Babbo, C.J., Friedlander, M. A. (1999) Gonadotropins induce higher active renin levels in the follicular fluid of normal and hyperstimulated cycles. *Gynecol Endocrinol*, **13**, 155-160.

Loukides, J. A., Loy, R. A., Edwards, R., Honig, J., Visintin, I. and Polan, M. L. (1990) Human follicular fluids contain tissue macrophages. *J Clin Endocrinol Metab*, **71**, 1363-1367.

Lucisano, A., Morandotti, G., Marana, R., Leone, F., Branca, G., Dell'Acqua, S. and Sanna, A. (1992) Chlamydial genital infections and laparoscopic findings in infertile women. *Eur J Epidemiol*, **8**, 645-649.

Lyons, R., Saridogan, E., Djahanbakhch, O. (2006) The reproductive significance of human Fallopian tube cilia. *Hum Reprod Update*, **12**, 363-372.

Makrigiannakis, A., Coukos, G., Christofidou-Solomidou, M., Montas, S. and Coutifaris, C. (2000) Progesterone is an autocrine/paracrine regulator of human granulosa cell survival *in vitro*. *Ann N Y Acad Sci*., **900**, 16-25.

Mann, K.G. (2003) Thrombin formation. *Chest*. **124**, 4S-10S.

Mardh, P., Baldetorp, B., Hakansson, C., Fritz, H., Westrom, L. (1979) Studies of ciliated epithelia of the human genital tract. 3. Mucociliary wave activity in organ cultures of human Fallopian tubes challenged with *Neisseria gonorrhoeae* and gonococcal endotoxin. *Br J Vener Dis*, **55**, 256-264.

Mardh, P. A. and Westrom, L. (1970) Tubal and cervical cultures in acute salpingitis with special reference to *Mycoplasma hominis* and t-strain mycoplasmas. *Br J Vener Dis*, **46**, 179-186.

Mardh, P. A. (2004) Tubal factor infertility, with special regard to chlamydial salpingitis. *Curr Opin Infect Dis*, **17**, 49-52.

Martin, D. H. (2008) Urethritis in Males. In Holmes, K. K. *et al*. (eds.) *Sexually Transmitted Diseases*. McGraw-Hill, New York.

Martin, H. L., Richardson, B. A., Nyange, P. M., Lavreys, L., Hillier, S. L., Chohan, B., Mandaliya, K., Ndinya-Achola, J. O., Bwayo, J. and Kreiss, J. (1999) Vaginal lactobacilli, microbial flora, and risk of human immunodeficiency virus type 1 and sexually transmitted disease acquisition. *J Infect Dis*, **180**, 1863-1868.

Martin, R., Soberon, N., Vaneechoutte, M., Florez, A. B., Vazquez, F. and Suarez, J. E. (2008) Characterisation of indigenous vaginal lactobacilli from healthy women as probiotic candidates. *Int Microbiol*, **11**, 261-266.

Martinez, M. A., Ovalle, A., Santa-Cruz, A., Barrera, B., Vidal, R. and Aguirre, R. (2001) Occurrence and antimicrobial susceptibility of *Ureaplasma parvum* (*Ureaplasma urealyticum* biovar 1) and *Ureaplasma urealyticum* (*Ureaplasma urealyticum* biovar 2) from patients with adverse pregnancy outcomes and normal pregnant women. *Scand J Infect Dis*, **33**, 604-610.

Mastroianni, L., Jr. (1999) The Fallopian tube and reproductive health. *J Pediatr Adolesc Gynecol*, **12**, 121-126.

Matalliotakis, I. M., Cakmak, H., Fragouli, Y., Kourtis, A., Arici, A. and Huszar, G. (2006) Increased IL-18 levels in seminal plasma of infertile men with genital tract infections. *Am J Reprod Immunol*, **55**, 428-433.

McClure, E. M. and Goldenberg, R. L. (2009) Infection and stillbirth. *Semin Fetal Neonatal Med*, **14**, 182-189.

McGee, Z. A., Stephens, D. S., Hoffman, L. H., Schlech, W. F., 3rd and Horn, R. G. (1983) Mechanisms of mucosal invasion by pathogenic Neisseria. *Rev Infect Dis*, **5**, S708-714.

McGowin, C. L., Popov, V. L. and Pyles, R. B. (2009) Intracellular *Mycoplasma genitalium* infection of human vaginal and cervical epithelial cells elicits distinct patterns of inflammatory cytokine secretion and provides a possible survival niche against macrophage-mediated killing. *BMC Microbiol*, **14**, 139-149.

McGregor, J. A., French, J. I., Richter, R., Vuchetich, M., Bachus, V., Seo, K., Hillier, S., Judson, F. N., McFee, J., Schoonmaker, J. (1990) Cervicovaginal microflora and pregnancy outcome: Results of a double-blind, placebo-controlled trial of erythromycin treatment. *Am J Obstet Gynecol*, **163**, 1580-1591.

Mehta, R. H., Sridhar, H., Vijay Kumar, B. R. and Anand Kumar, T. C. (2002) High incidence of oligozoospermia and teratozoospermia in human semen infected with the aerobic bacterium *Streptococcus faecalis*. *Reprod Biomed Online*, **5**, 17-21.

Meier, B. and Habermehl, G. G. (1990) Evidence for superoxide dismutase and catalase in mollicutes and release of reactive oxygen species. *Arch Biochem Biophys*, **277**, 74-79.

Meirik O. (2007) Intrauterine devices - upper and lower genital tract infections. *Contraception*, **75**, S41-7.

Mendoza, C., Ruiz-Requena, E., Ortega, E., Cremades, N., Martinez, F., Bernabeu, R., Greco, E. and Tesarik, J. (2002) Follicular fluid markers of oocyte developmental potential. *Hum Reprod*, **17**, 1017-1022.

Meyer, T. F. (1999) Pathogenic neisseriae: Complexity of pathogen-host cell interplay. *Clin Infect Dis*, **28**, 433-441.

Mikami, K., TakahashiI, H., Kimura, M., Isozaki, M., Izuchi, K., Shibata, R., Sudo, N., Matsumoto, H., Koga, Y., (2009) Influence of maternal bifidobacteria on the establishment of bifidobacteria colonising the gut in infants. *Pediatric Research*, **65** 669-674.

Mikamo, H., Ninomiya, M., Tamaya, T., Kim, S. W., Nah, M. Y., Yeum, C. H., Kim, N. H., Choi, H. S., Juhng, S. W., Choi, K. C., Toy, E. C., Scerpella, E. G., Riggs, J. W., Kostelnik, F. V., Fremount, H. N., Saw, E. C., Smale, L. E., Einstein, H., Huntington, R. W., Jr., Craig, M. W., Davey, W. N., Green, R. A. and Plaut, A. (2003) Tubo-ovarian abscess caused by *Candida glabrata* in a febrile neutropenic patient. *J Infect Chemother*, **9**, 257-259.

Mittwoch, U. (1975) Letter: Lateral asymmetry and gonadal differentiation. *Lancet*, **1**, 401-402.

Moller, B. R., Taylor-Robinson, D., Furr, P. M., Toft, B. and Allen, J. (1985) Serological evidence that chlamydiae and mycoplasmas are involved in infertility of women. *J Reprod Fertil*, **73**, 237-240.

Monds, R. D. and O'Toole, G. A. (2009) The developmental model of microbial biofilms: Ten years of a paradigm up for review. *Trends Microbiol*, **17**, 73-87.

Montagut, J. M., Lepretre, S., Degoy, J. and Rousseau, M. (1991) *Ureaplasma* in semen and IVF. *Hum Reprod*, **6**, 727-729.

Monteleone, P., Giovanni Artini, P., Simi, G., Casarosa, E., Cela, V. and Genazzani, A. R. (2008) Follicular fluid VEGF levels directly correlate with perifollicular blood flow in normoresponder patients undergoing IVF. *J Assist Reprod Genet*, **25**, 183-186.

Moore, D. E., Soules, M. R., Klein, N. A., Fujimoto, V. Y., Agnew, K. J. and Eschenbach, D. A. (2000) Bacteria in the transfer catheter tip influence the live-birth rate after *in vitro* fertilisation. *Fertil Steril*, **74**, 1118-1124.

Moore, P. (2000) Research needed into effects of IVF. *Brit Med J*, **320**, 960.

Mori, H., Hanabayashi, T., Yamada, Y. and Tamaya, T. (1990) Decrease in interferon-gamma production by peripheral blood mononuclear cells in patients with uterine cervical cancer. *J Clin Immunol*, **10**, 45-51.

Morrison, R. P., Belland, R. J., Lyng, K. and Caldwell, H. D. (1989) Chlamydial disease pathogenesis. The 57-kD chlamydial hypersensitivity antigen is a stress response protein. *J Exp Med*, **170**, 1271-1283.

Motro, B., Itin, A., Sachs, L. and Keshet, E. (1990) Pattern of interleukin-6 gene expression *in vivo* suggests a role for this cytokine in angiogenesis. *Proc Natl Acad Sci U S A*, **87**, 3092-3096.

Mukaida, T., Nakamura, S., Tomiyama, T., Wada, S., Oka, C., Kasai, M. and Takahashi, K. (2003) Vitrification of human blastocysts using cryoloops: Clinical outcome of 223 cycles. *Hum Reprod*, **18**, 384-391.

Munne, S., Weier, H. U., Stein, J., Grifo, J. and Cohen, J. (1993) A fast and efficient method for simultaneous X and Y *in situ* hybridization of human blastomeres. *J Assist Reprod Genet*, **10**, 82-90.

Naessens, A., Foulon, W., Debrucker, P., Devroey, P. and Lauwers, S. (1986) Recovery of microorganisms in semen and relationship to semen evaluation. *Fertil Steril*, **45**, 101-105.

Ness, R. B., Soper, D. E., Holley, R. L., Peipert, J., Randall, H., Sweet, R. L., Sondheimer, S. J., Hendrix, S. L., Amortegui, A., Trucco, G., Bass, D. C. and Kelsey, S. F. (2001) Hormonal and barrier contraception and risk of upper genital tract disease in the PID evaluation and clinical health (PEACH) study. *Am J Obstet Gynecol*, **185**, 121-127.

Ness, R. B., Kip, K. E., Hillier, S. L., Soper, D. E., Stamm, C. A., Sweet, R. L., Rice, P. and Richter, H. E. (2005) A cluster analysis of bacterial vaginosis-associated microflora and pelvic inflammatory disease. *Am J Epidemiol*, **162**, 585-590.

Ness, R. B., Soper, D. E., Richter, H. E., Randall, H., Peipert, J. F., Nelson, D. B., Schubeck, D., McNeeley, S. G., Trout, W., Bass, D. C., Hutchison, K., Kip, K. and Brunham, R. C. (2008) Chlamydia antibodies, chlamydia heat shock protein, and adverse sequelae after pelvic inflammatory disease: The PID evaluation and clinical health (PEACH) study. *Sex Transm Dis*, **35**, 129-135.

Ng, S. C., Gilman-Sachs, A., Thaker, P., Beaman, K. D., Beer, A. E. and Kwak-Kim, J. (2002) Expression of intracellular Th1 and Th2 cytokines in women with recurrent spontaneous abortion, implantation failures after IVF/ET or normal pregnancy. *Am J Reprod Immunol*, **48**, 77-86.

Norman, R. J. and Brannstrom, M. (1996) Cytokines in the ovary: Pathophysiology and potential for pharmacological intervention. *Pharmacol Ther*, **69**, 219-236.

Nugent, R. P., Krohn, M. A. and Hillier, S. L. (1991) Reliability of diagnosing bacterial vaginosis is improved by a standardized method of gram stain interpretation. *J Clin Microbiol*, **29**, 297-301.

Nunez-Calonge, R., Caballero, P., Redondo, C., Baquero, F., Martinez-Ferrer, M. and Meseguer, M. A. (1998) *Ureaplasma urealyticum* reduces motility and induces membrane alterations in human spermatozoa. *Hum Reprod*, **13**, 2756-2761.

Oakeshott, P., Hay, P., Taylor-Robinson, D., Hay, S., Dohn, B., Kerry, S. and Jensen, J. S. (2004) Prevalence of *Mycoplasma genitalium* in early pregnancy and relationship between its presence and pregnancy outcome. *Brit J Obstet Gynecol*, **111**, 1464-1467.

Oakeshott, P., Hay, P., Hay, S., Steinke, F., Rink, E., Kerry, S. (2002) Association between bacterial vaginosis or chlamydial infection and miscarriage before 16 weeks' gestation: Prospective community based cohort study. *Primary Care*, **325**, 1334-1338.

Ogasawara, M. S., Aoki, K., Aoyama, T., Katano, K., Iinuma, Y., Ozaki, Y. and Suzumori, K. (2000) Elevation of transforming growth factor β1 is associated with recurrent miscarriage. *J Clin Immunol*, **20**, 453-457.

Oger, P., Bulla, R., Tedesco, F., Portier, A., Dubanchet, S., Bailly, M., Wainer, R., Chaouat, G. and Lédée, N. (2009) Higher interleukin-18 and mannose-binding lectin are present in uterine lumen of patients with unexplained infertility. *Reprod Biomed Online*, **19**, 591-598.

Ohman, H., Tiitinen, A., Halttunen, M., Paavonen, J. and Surcel, H. M. (2011) Cytokine gene polymorphism and *Chlamydia trachomatis*-specific immune responses. *Hum Immunol*, **72**, 278-282.

Onderdonk, A. B., Delaney, M. L., DuBois, A. M., Allred, E. N. and Leviton, A. (2008a) Detection of bacteria in placental tissues obtained from extremely low gestational age neonates. *Am J Obstet Gynecol*, **198**, 110 e111-117.

Onderdonk, A. B., Hecht, J. L., McElrath, T. F., Delaney, M. L., Allred, E. N. and Leviton, A. (2008b) Colonisation of second-trimester placenta parenchyma. *Am J Obstet Gynecol*, **199**, 52 e51-52.

Orengo, A.M., Fabbi, M., Miglietta, L., Andreani, C., Bruzzone, M., Puppo, A., Cristoforoni, P., Centurioni, M.G., Gualco, M., Salvi, S., Boccardo, S., Truini, M., Piazza, T., Canevari, S., Mezzanzanica, D., Ferrini, S. (2011) Interleukin-18, a biomarker of human ovarian carcinoma, is predominantly released as biologically inactive precursor. *Int J Cancer*, **129**, 1116-1125

Paavonen, J., Westrom, L. and Eschenbach, D. (2008) Pelvic Inflammatory disease. In Holmes, K. K. *et al*. (eds.) *Sexually Transmitted Diseases*. McGraw-Hill, New York.

Pacora, P., Chaiworapongsa, T., Maymon, E., Kim, Y. M., Gomez, R., Yoon, B. H., Ghezzi, F., Berry, S. M., Qureshi, F., Jacques, S. M., Kim, J. C., Kadar, N. and Romero, R. (2002) Funisitis and chorionic vasculitis: The histological counterpart of the fetal inflammatory response syndrome. *J Matern Fetal Neonatal Med*, **11**, 18-25.

Palermo, G., Joris, H., Devroey, P. and Van Steirteghem, A. C. (1992) Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet*, **340**, 17-18.

Palmer, H. M., Gilroy, C. B., Claydon, E. J. and Taylor-Robinson, D. (1991) Detection of *Mycoplasma genitalium* in the genitourinary tract of women by the polymerase chain reaction. *Int J STD AIDS*, **2**, 261-263.

Pamp, S. J., Gjermansen, M., Johansen, H. K. and Tolker-Nielsen, T. (2008) Tolerance to the antimicrobial peptide colistin in *Pseudomonas aeruginosa* biofilms is linked to metabolically active cells, and depends on the pmr and mexab-oprm genes. *Mol Microbiol*, **68**, 223-240.

Pamp, S. J., Sternberg, C. and Tolker-Nielsen, T. (2009) Insight into the microbial multicellular lifestyle via flow-cell technology and confocal microscopy. *Cytometry A*, **75**, 90-103.

Pamp, S. J., Gjermansen, M., Johansen, H.K., Tolker-Nielsen, T. (2008) Tolerance to the antimicrobial peptide colisitin in *Pseudomonas aeruginosa* biofilms is linked to metabolically active cells, and depends on the pmr and mexab_oprm genes. *Mol Microbiol*, **68**, 223-240.

Pan, C. J., Wu, H.L., Kuo, S.F., Kao, J. H., Tseng, T. C., Liu, C. H., Chen, P. J., Liu, C. J. and Chen, D.S. (2011) Serum interleukin-6 level correlates with outcomes of acute exacerbation of chronic hepatitis B. *Hepatol Int*. Epub Jul 16.

Parsons, L. S. and Simmons, C. (1978) *Tuberculosis and Other Specific Infections That Produce Pelvic Inflammation.* 2nd edn, W.B. Saunders Co., Philadelphia.

Pascual, A., Jaton, K., Ninet, B., Bille, J. and Greub, G. (2010) New diagnostic real-time PCR for specific detection of *Mycoplasma hominis* DNA. *Int J Microbiol*, **2010**, 317512.

Patten, E. (1987) Immunohematologic diseases. *J Amer Medical Assoc*, **258**, 2945-2951.

Patterson, J. L., Stull-Lane, A., Girerd, P. H. and Jefferson, K. K. (2009) Analysis of adherence, biofilm formation, and cytotoxicity suggest a greater virulence potential of *Gardnerella vaginalis* relative to other bacterial vaginosis-associated anaerobes. *Microbiol*. **156**, 392-399

Pellicer, A., Garrido, N., Albert, C., Navarro, J., Remohi, J. and Simon, C. (1999) Cytokines in older patients undergoing *in vitro* fertilisation: The relationship to the response to controlled ovarian hyperstimulation. *J Assist Reprod Genet*, **16**, 247-252.

Periasamy, S. and Kolenbrander, P. E. (2009) Mutualistic biofilm communities develop with *Porphyromonas gingivalis* and initial, early, and late colonizers of enamel. *J Bacteriol*, **191**, 6804-6811.

Pertel, P., and Spear, P. (2008) Biology of herpesviruses. In Holmes, K. K. *et al*. (eds.) *Sexually Transmitted Diseases*. McGraw-Hill, New York.

Pertel, P., and Spear, P. (1999) Biology of herpesviruses. In Holmes, K. K. *et al*. (eds.) *Sexually Transmitted Diseases*. McGraw-Hill, New York.

Petitbarat, M., Serazin, V., Dubanchet, S., Wayner, R., de Mazancourt, P., Chaouat, G. and Lédée, N. (2010) Tumor necrosis factor-like weak inducer of apoptosis (TWEAK)/fibroblast growth factor inducible-14 might regulate the effects of interleukin-18 and 15 in the human endometrium. *Fertil Steril*, **94**, 1141-1143.

Pinero-Sagredo, E., Nunes, S., de Los Santos, M. J., Celda, B. and Esteve, V. (2010) NMR metabolic profile of human follicular fluid. *NMR Biomed*, **23**, 485-495.

Pisa, P., Halapi, E., Pisa, E. K., Gerdin, E., Hising, C., Bucht, A., Gerdin, B. and Kiessling, R. (1992) Selective expression of interleukin-10, interferon γ, and granulocyte-macrophage colony-stimulating factor in ovarian cancer biopsies. *Proc Natl Acad Sci U S A*, **89**, 7708-7712.

Plaut, A. (1950) Human infection with *Cryptococcus glabratus*; report of case involving uterus and Fallopian tube. *Am J Clin Pathol*, **20**, 377-380.

Polan, M. L., Loukides, J., Nelson, P., Carding, S., Diamond, M., Walsh, A. and Bottomly, K. (1989) Progesterone and oestradiol modulate interleukin-1 βmessenger ribonucleic acid levels in cultured human peripheral monocytes. *J Clin Endocrinol Metab*, **69**, 1200-1206.

Population Council. (2003) Reproductive tract infections: An introductory overview. http://www.popcouncil.org/pdfs/RTIFacsheetsRev.pdf

Potashnik, G., Insler, V. and Meizner, L (1987) Frequence, sequence, and side of ovulation in women menstruating normally. *Br Med J (Clin Res Ed)*, **294**, 219.

Powell, C. R., Allshouse, M., Bethel, K. J. and Mevorach, R. A. (1998) Invasive aspergillosis of the scrotum. *J Urol*, **159**, 1306-1308.

Price, F. V., Chambers, S. K., Chambers, J. T., Carcangiu, M. L., Schwartz, P. E., Kohorn, E. I., Stanley, E. R. and Kacinski, B. M. (1993) Colony-stimulating factor-1 in primary ascites of ovarian cancer is a significant predictor of survival. *Am J Obstet Gynecol*, **168**, 520-527.

Price, E, P., Smith, H., Huygens, F. and Giffard, P.M. (2007) High-resolution DNA melt curve analysis of the clustered, regularly interspaced short-palindromic-repeat locus of *Campylobacter jejuni*. *Appl Environ Microbiol*, 73, 3431-3436.

Pruthi, V., Al-Janabi, A. and Pereira, B. J. (2003) Characterisation of biofilm formed on intrauterine devices. *Indian J Med Microbiol*, **21**, 161-165.

Quayle, A. J., Xu, C., Tucker, L., and Anderson, D. J. (1998) The case against an association between HIV-1 and sperm: molecular evidence. *J Reprod Immunol*, ***41***, 127-136.

Quinn, P. A., Rubin, S., Nocilla, D. M., Read, S. E. and Chipman, M. (1983) Serological evidence of *Ureaplasma urealyticum* infection in neonatal respiratory disease. *Yale J Biol Med*, **56**, 565-572.

Quinn, P. A., Butany, J., Taylor, J. and Hannah, W. (1987) Chorioamnionitis: Its association with pregnancy outcome and microbial infection. *Am J Obstet Gynecol*, **156**, 379-387.

Quirk, S. M., Cowan, R.G. and Harman, R. M. (2006) The susceptibility of granulosa cells to apoptosis is influenced by oestradiol and the cell cycle. *J Endocrinol*, **189**, 441-453.

Ralph, S. G., Rutherford, A. J. and Wilson, J. D. (1999) Influence of bacterial vaginosis on conception and miscarriage in the first trimester: Cohort study. *Brit Med J*, **319**, 220-223.

Rasmussen, S. J., Eckmann, L., Quayle, A. J., Shen, L., Zhang, Y. X., Anderson, D. J., Fierer, J., Stephens, R. S., Kagnoff, M. F. (1997) Secretion of pro-inflammatory cytokines by epithelial cells in response to Chlamydia infection suggests a central role for epithelial cells in chlamydial pathogenesis. *J Clin Invest*, **99**, 77-87.

Rayner, C. R., Ioannides-Demos, L. L., Brien, J. A., Liolios, L. L. and Spicer, W. J. (1998) Initial concentration-time profile of gentamicin determines efficacy against *Enterobacter cloacae* ATCC 13047. *Antimicrob Agents Chemother*, **42**, 1370-1374.

Raz, R. and Stamm, W.E. (1993) A controlled trial of intravaginal estriol in postmenopausal women with recurrent urinary tract infections. *N Engl J Med* **329**, 753-756.

Reichart, M., Kahane, I. and Bartoov, B. (2000) *In vivo* and *in vitro* impairment of human and ram sperm nuclear chromatin integrity by sexually transmitted *Ureaplasma urealyticum* infection. *Biol Reprod*, **63**, 1041-1048.

Ribardo, D. A., Crowe, S. E., Kuhl, K. R., Peterson, J. W. and Chopra, A. K. (2001) Prostaglandin levels in stimulated macrophages are controlled by phospholipase A2-activating protein and by activation of phospholipase C and D. *J Biol Chem*, **276**, 5467-5475.

Richards, J. S., Sharma, S. C., Falender, A. E. and Lo, Y. H. (2002) Expression of fkhr, fkhrl1, and afx genes in the rodent ovary: Evidence for regulation by IGF-I, oestrogen, and the gonadotropins. *Mol Endocrinol*, **16**, 580-599.

Riggs, M., Klebanoff, M., Nansel, T., Zhang, J., Schwebke, J. and Andrews, W. (2007) Longitudinal association between hormonal contraceptives and bacterial vaginosis in women of reproductive age. *Sex Transm Dis*, **34**, 954-959.

Robertson, S. A., Guerin, L. R., Moldenhauer, L. M. and Hayball, J. D. (2009) Activating T regulatory cells for tolerance in early pregnancy - the contribution of seminal fluid. *J Reprod Immunol*, **83**, 109-116.

Roach, L. E., Petrik, J. J., Plante, L., LaMarre, J. and Gentry, P.A. (2002) Thrombin generation and presence of thrombin receptor in ovarian follicles. *Biol Reprod*, **66**, 1350-1358.

Rodin, D. M., Larone, D. and Goldstein, M. (2003) Relationship between semen cultures, leukospermia, and semen analysis in men undergoing fertility evaluation. *Fertil Steril*, **79**, 1555-1558.

Romero, R. and Mazor, M. (1988) Infection and preterm labor. *Clin Obstet Gynecol*, **31**, 553-584.

Romero, R., Gomez, R., Chaiworapongsa, T., Conoscenti, G., Kim, J. C. and Kim, Y. M. (2001) The role of infection in preterm labour and delivery. *Paediatr Perinat Epidemiol*, **15**, 41-56.

Romero, R., Chaiworapongsa, T. and Espinoza, J. (2003) Micronutrients and intrauterine infection, preterm birth and the fetal inflammatory response syndrome. *J Nutr*, **133**, 1668S-1673S.

Romero, R., Gotsch, F., Pineles, B. and Kusanovic, J. P. (2007) Inflammation in pregnancy: Its roles in reproductive physiology, obstetrical complications, and fetal injury. *Nutr Rev*, **65**, S194-202.

Romero, R., Schaudinn, C., Kusanovic, J. P., Gorur, A., Gotsch, F., Webster, P., Nhan-Chang, C. L., Erez, O., Kim, C. J., Espinoza, J., Goncalves, L. F., Vaisbuch, E., Mazaki-Tovi, S., Hassan, S. S. and Costerton, J. W. (2008) Detection of a microbial biofilm in intraamniotic infection. *Am J Obstet Gynecol*, **198**, 135 e131-135.

Ryckman, K. K., Williams, S. M. and Kalinka, J. (2008) Correlations of selected vaginal cytokine levels with pregnancy-related traits in women with bacterial vaginosis and mycoplasmas. *J Reprod Immunol*, **78**, 172-80.

Saffos, S. O. and Rhatigan, R. M. (1977) Unilateral salpingitis due to *Enterobius vermicularis*. *Am J Clin Pathol*, **67**, 296-299.

Sager, R., Anisowicz, A., Pike, M. C., Beckmann, P. and Smith, T. (1992) Structural, regulatory, and functional studies of the gro gene and protein. *Cytokines*, **4**, 96-116.

Saini, S., Gupta, N., Batra, G. and Arora, D. R. (2003) Role of anaerobes in acute pelvic inflammatory disease. *Indian J Med Microbiol*, **21**, 189-192.

Saladin, K. (2007) *Anatomy and Physiology: The unity of form and function*, McGraw-Hill, New York.

Salcido, R. (2007) What is bioburden? The link to chronic wounds. *Adv Skin Wound Care*, **20**, 368-369.

Salmassi, A., Lü, S., Hedderich, J., Oettinghaus, C., Jonat, W. And Mettler, L. (2001) Interaction of interleukin-6 on human granulosa cell steroid secretion. *J Endocrinol*, **170**, 471-478.

Salmassi, A. S., A.G., Schaefer, S., Koch, K., Hedderich, J., Jonat, W. and Mettler, L. (2005) Is granulocyte colony-stimulating factor level predictive for human IVF outcome? *HumReprod*, **20**, 2434–2440.

Saltes, B., Molo, M., Binor, Z., Radwanska, E. (1995) Bacterial contamination after trans-vaginal aspiration of oocytes. *J Assist Reprod Genetics*, **12**, 657-658.

Sanchez, M. C., Llama-Palacios, A., Blanc, V., Leon, R., Herrera, D. and Sanz, M. (2011) Structure, viability and bacterial kinetics of an *in vitro* biofilm model using six bacteria from the subgingival microbiota. *J Periodontal Res*, **46**, 252-260.

Sanchez, R., Villagran, E., Concha, M. and Cornejo, R. (1989) Ultrastructural analysis of the attachment sites of *Escherichia coli* to the human spermatozoon after *in vitro* migration through oestrogenic cervical mucus. *Int J Fertil*, **34**, 363-367.

Sarrell, P. M. a. Pruett, K. A. (1968) Symptomatic gonorrhoea during pregnancy. *Obstet gynecol*, **32**, 670.

Satta, A., Stivala, A., Garozzo, A., Morello, A., Perdichizzi, A., Vicari, E., Salmeri, M. and Calogero, A. E. (2006) Experimental *Chlamydia trachomatis* infection causes apoptosis in human sperm. *Hum Reprod*, **21**, 134-137.

Saunders, S., Bocking, A., Challis, J. and Reid, G. (2007) Effect of Lactobacillus challenge on gardnerella vaginalis biofilms. *Colloids Surf B Biointerfaces*, **55**, 138-142.

Sautter, R. L. and Brown, W. J. (1980) Sequential vaginal cultures from normal young women. *J Clin Microbiol*, **11**, 479-484.

Saw, E. C., Smale, L. E., Einstein, H., Huntington, R. W., Jr., Craig, M. W., Davey, W. N., Green, R. A. and Plaut, A. (1975) Female genital coccidioidomycosis. *Obstet Gynecol*, **45**, 199-202.

Scarpellini, F. and Sbracia, M. (2009) Use of granulocyte colony-stimulating factor for the treatment of unexplained recurrent miscarriage: A randomised controlled trial. *Hum Reprod*, **24**, 2703-2708.

Schachter, J. (1999) Biology of *Chlamydia trachomatis*. In Holmes, K. K. *et al.* (eds.) *Sexually Transmitted Diseases*. McGraw-Hill, New York.

Schlesinger, P., Duray, P., Burke, B. (1985) Maternal-fetal transmission of the lyme disease spirochete, *Borrelia burgdorferi*. *Ann Intern Med*, **103**, 67-68.

Schultz, G. S., Sibbald, R.G., Falanga, V., Ayello, E. A., Dowsett, C., Harding, K., Romanelli, M., Stacey, M. C., Teot, L. and Vanscheidt, W. (2003) Wound bed preparation: a systematic approach to wound management. *Wound Repair Regen*, **11**, S1-28.

Serono International (2006) *Atlas of Assisted Reproduction*, Serono International, Geneva.

Shaio, M. F., Lin, P. R., Liu, J. Y. and Yang, K. D. (1995) Generation of interleukin-8 from human monocytes in response to *Trichomonas vaginalis* stimulation. *Infect Immun*, **63**, 3864-3870.

Shalika, S., Dugan, K., Smith, R. D. and Padilla, S. L. (1996) The effect of positive semen bacterial and Ureaplasma cultures on *in vitro* fertilisation success. *Hum Reprod*, **11**, 2789-2792.

Sharpe, K., Karovitch, A., Claman, P., Suh, K. (2006) Transvaginal oocyte retrieval for in virto fertilisation complicated by ovarian abscess during pregnancy. *Fertil Steril*, **86**, 11-13.

Shepard, M. C., Lunceford, C.D., Ford, D.K., Purcell, R.H., Taylor-Robinson, D., Razin, S., Black, F.T (1974) *Ureaplasma urealyticum* gen. Nov., sp nov: Proposed nomenclature for the human T strain (T-strain) mycoplasmas. *Int J System Bacteriol*, **24**, 160-174.

Shimada, H., Kasakura, S., Shiotani, M., Nakamura, K., Ikeuchi, M., Hoshino, T., Komatsu, T., Ihara, Y., Sohma, M., Maeda, Y., Matsuura, R., Nakamura, S., Hine, C., Ohkura, N. and Kato, H. (2001) Hypocoagulable state of human preovulatory ovarian follicular fluid: Role of sulfated proteoglycan and tissue factor pathway inhibitor in the fluid. *Biol Reprod*, **64**, 1739-1745.

Shimada, S., Iwabuchi, K., Kato, E. H., Morikawa, M., Sakuragi, N., Onoe, K., Minakami, H. and Yamada, H. (2003) No difference in natural-killer-T cell population, but Th2/Tc2 predominance in peripheral blood of recurrent aborters. *Am J Reprod Immunol*, **50**, 334-339.

Siddiqui, A. R. and Bernstein, J. M. (2010) Chronic wound infection: Facts and controversies. *Clin Dermatol*, **28**, 519-526.

Silver, H. M., Sperling, R. S., St Clair, P. J. and Gibbs, R. S. (1989) Evidence relating bacterial vaginosis to intraamniotic infection. *Am J Obstet Gynecol*, **161**, 808-812.

Simhan, H. N., Anderson, B. L., Krohn, M.A., Heine, R. P., Martinez de Tejada, B., Landers, D. V. and Hillier, S. L. (2007) Host immune consequences of asymptomatic *Trichomonas vaginalis* infection in pregnancy. *Am J Obstet Gynecol*, **196**, e1-5.

Simmons, W. L., Bolland, J. R., Daubenspeck, J. M. and Dybvig, K. (2007) A stochastic mechanism for biofilm formation by *Mycoplasma pulmonis*. *J Bacteriol*, **189**, 1905-1913.

Singer, A. J., Kubak, B. and Anders, K. H. (1998) Aspergillosis of the testis in a renal transplant recipient. *Urology*, **51**, 119-121.

Slobod, K., and Sixbey, J. (1999) Epstein-barr virus infection. In Holmes, K. K. *et al*. (eds.) *Sexually Transmitted Diseases*. McGraw-Hill, New York.

Sluss, P. M., Fletcher, P. W. and Reichert, L. E., Jr. (1983) Inhibition of 125i-human follicle-stimulating hormone binding to receptor by a low molecular weight fraction of bovine follicular fluid: Inhibitor concentration is related to biochemical parameters of follicular development. *Biol Reprod*, **29**, 1105-1113.

Sluss, P. M., Lee, K., Mattox, J. H., Smith, P. C., Graham, M. C. and Partridge, A. B. (1994) Oestradiol and progesterone production by cultured granulosa cells cryopreserved from *in vitro* fertilisation patients. *Eur J Endocrinol*, **130**, 259-264.

Smale, L. E., and Waechter, K.G. (1970) Dissemination of coccidioidomycosis in pregnancy. *Am J Obstet Gynecol*, **107**, 356-359.

Smith, K. S. and Ferry, J. G. (2000) Prokaryotic carbonic anhydrases. *FEMS Microbiol Rev*, **24**, 335-366.

Sobel, J. (1999) Vulvovaginal candidiasis. In Holmes, K., Sparling, P., Mardh, P., Lemon, S., Stamm, W., Piot, P., Wasserheit, J. (eds.) *Sexually Transmitted Diseases*. McGraw-Hill, New York.

Sonnex, C. (1998) Influence of ovarian hormones on urogenital infection. *Sex Transm Infect*, **74**, 11-19.

Sorbye, I. K., Jerve, F. and Staff, A. C. (2005) Reduction in hospitalised women with pelvic inflammatory disease in Oslo over the past decade. *Acta Obstet Gynecol Scand*, **84**, 290-296.

Spaine, D., Mamizuka, E., Cedenho, A., Srougi, M. (2000) Microbiologic aerobic studies on normal male urethra. *Adult Urol*, **56**, 207-210.

Spandorfer, S. D., Neuer, A., Giraldo, P. C., Rosenwaks, Z. and Witkin, S. S. (2001) Relationship of abnormal vaginal flora, pro-inflammatory cytokines and idiopathic infertility in women undergoing IVF. *J Reprod Med*, **46**, 806-810.

Spandorfer, S. D., Neuer, A., Liu, H. C., Rosenwaks, Z. and Witkin, S. S. (2003) Involvement of interleukin-1 and the interleukin-1 receptor antagonist in *in vitro* embryo development among women undergoing *in vitro* fertilisation-embryo transfer. *J Assist Reprod Genet*, **20**, 502-505.

Spandorfer, S. D., Bongiovanni, A. M., Fasioulotis, S., Rosenwaks, Z., Ledger, W. J., and Witkin, S. S. (2006) Prevalence of cervical human papillomavirus in women undergoing *in vitro* fertilisation and association with outcome. *Fertil Steril*, ***86***, 765-767.

Spence, M. R., Blanco, L. J., Patel, J. and Brockman, M. T. (1982) A comparative evaluation of vaginal, cervical and peritoneal flora in normal, healthy women: A preliminary report. *Sex Transm Dis*, **9**, 37-40.

Speroff, L. and Fritz, M. A. (2005) *Clinical Gynecologic Endocrinology and Infertility*. 7th edn, Lippincott Williams and Wilkins.

Spicer, W. (2000) *Clinical Bacteriology, Mycology and Parasitology*, Churchill Livingston, London.

Splichalova, A., Trebichavsky, I., Muneta, Y., Mori, Y. and Splichal, I. (2005) Effect of bacterial virulence on IL-18 expression in the amnion infected with *Escherichia coli*. *Am J Reprod Immunol*, **53**, 255-260.

Spurbeck, R. R. and Arvidson, C. G. (2008) Inhibition of *Neisseria gonorrhoeae* epithelial cell interactions by vaginal lactobacillus species. *Infect Immun*, **76**, 3124-3130.

Srinivasan, U., Misra, D., Marazita, M. L. and Foxman, B. (2009) Vaginal and oral microbes, host genotype and preterm birth. *Med Hypotheses*, **73**, 963-975.

Stagey, C., Munday, P., Taylor-Robinson, D., Thomas, B., Gilchrist, C., Ruck, F., Isdn, C. and Beard, R. (1992) A longitudinal study of pelvic inflammatory disease. *Br J Obstet Gynaecol*, **99**, 994-999.

Stanley, M.A. (2001) Human papillomavirus and cervical carcinogenesis. *Best Pract Res Clin Obstet Gynaecol*, **15**, 663-676.

Stepanovic, S., Djukic, S., Veljkovic, M., Arsic, B., Garalejic, E., Ranin, L. (2003) Antimicrobial activity of human follicular fluids. *Gynecol Obstet Investig*, **56**, 173-178.

Stephenson, J., Hopwood, J., Babiker, A., Copas, A. and Vickers, M. (2003) Recent pilot studies of chlamydia screening. *Sex Transm Infect*, **79**, 352.

Stewart, P. S., Grab, L. and Diemer, J. A. (1998) Analysis of biocide transport limitation in an artificial biofilm system. *J Appl Microbiol*, **85**, 495-500.

Stewart, P. S. and Costerton, J. W. (2001) Antibiotic resistance of bacteria in biofilms. *Lancet*, **358**, 135-138.

Stone, S. C. (1980) Complications and pitfalls of artificial insemination. *Clin Obstet Gynecol*, **23**, 667-682.

Sunderkotter, C., Steinbrink, K., Goebeler, M., Bhardwaj, R. and Sorg, C. (1994) Macrophages and angiogenesis. *J Leukoc Biol*, **55**, 410-422.

Svenstrup, H. F., Fedder, J., Abraham-Peskir, J., Birkelund, S. and Christiansen, G. (2003) *Mycoplasma genitalium* attaches to human spermatozoa. *Hum Reprod*, **18**, 2103-2109.

Sweet, R. L., Blankfort-Doyle, M., Robbie, M. O. and Schacter, J. (1986) The occurrence of chlamydial and gonococcal salpingitis during the menstrual cycle. *J Amer Med Assoc*, **255**, 2062-2064.

Swenson, C. E., Donegan, E. and Schachter, J. (1983) *Chlamydia trachomatis*-induced salpingitis in mice. *J Infect Dis*, **148**, 1101-1107.

Swenson, C. E. and Schachter, J. (1984) Infertility as a consequence of chlamydial infection of the upper genital tract in female mice. *Sex Transm Dis*, **11**, 64-67.

Swidsinski, A., Mendling, W., Loening-Baucke, V., Ladhoff, A., Swidsinski, S., Hale, L. P. and Lochs, H. (2005) Adherent biofilms in bacterial vaginosis. *Obstet Gynecol*, **106**, 1013-1023.

Symmers, W. S. C. (1950) Pathology of oxyuriosis with special reference to granulomas due to presence of *Enterobius vermicularis* and its ova in tissues. *Arch Pathol*, **50**, 475-516.

Sznurkowski, J. J. and Emerich, J. (2008) Endometriomas are more frequent on the left side. *Acta Obstet Gynecol Scand*, **87**, 104-106.

Tadros, H., Chretien, M. and Mbikay, M. (2001) The testicular germ-cell protease pc4 is also expressed in macrophage-like cells of the ovary. *J Reprod Immunol*, **49**, 133-152.

Tafari, N., Ferede, A., Girmai, M., Mullen, S. and Aberra, T. R. (1976) Neonatal septicaemia. *Ethiop Med J*, **14**, 169-177.

Talkington, D. F., Davis, J. K., Canupp, K. C., Garrett, B. K., Waites, K. B., Huster, G. A. and Cassell, G. H. (1991) The effects of three serotypes of *Ureaplasma urealyticum* on spermatozoal motility and penetration *in vitro*. *Fertil Steril*, **55**, 170-176.

Tamura, H., Takasaki, A., Miwa, I., Taniguchi, K., Maekawa, R., Asada, H., Taketani, T., Matsuoka, A., Yamagata, Y., Shimamura, K., Morioka, H., Ishikawa, H., Reiter, R. J. and Sugino, N. (2008) Oxidative stress impairs oocyte quality and melatonin protects oocytes from free radical damage and improves fertilisation rate. *J Pineal Res*, **44**, 280-287.

Tang, V.A., and Rosenthal, K.L. (2010) Intra-vaginal infection with herpes simplex virus type-2 (HSV-2) generates a functional effector memory T cell population that persists in the murine genital tract. *J Reprod Immunol*, **87**, 39-44.

Taylor-Robinson, A. W., Borriello, S. P. and Taylor-Robinson, D. (1993 a) Identification and preliminary characterisation of a cytotoxin isolated from *Mobiluncus* spp. *Int J Exp Pathol*, **74**, 357-366.

Taylor-Robinson, D. and Furr, P. M. (1993 b) Models of infection due to mycoplasmas, including *Mycoplasma fermentans*, in the genital tract and other sites in mice. *Clin Infect Dis*, **17**, S280-282.

Taylor-Robinson, D., Gilroy, C. B. and Hay, P. E. (1993 c) Occurrence of *Mycoplasma genitalium* in different populations and its clinical significance. *Clin Infect Dis*, **17**, S66-68.

Taylor-Robinson, D. (1996) Infections due to species of mycoplasma and ureaplasma: An update. *Clin Infect Dis*, **23**, 671-682.

Taylor, S. and Frydman, R. (1996) [Hysteroscopy and sperm infection]. *Contracept Fertil Sex*, **24**, 549-551. Abstract only

Taylor, S. N. (2005) *Mycoplasma genitalium. Curr Infect Dis Rep*, **7**, 453-457.

te Velde, E. R. and Cohlen, B. J. (1999) The management of infertility. *N Engl J Med*, **340**, 224-226.

Thomas, L. V., Cravioto, A., Scotland, S. M. and Rowe, B. (1982) New fimbrial antigenic type (e8775) that may represent a colonisation factor in enterotoxigenic *Escherichia coli* in humans. *Infect Immun*, **35**, 1119-1124.

Tolockiene, E., Morsing, E., Holst, E., Herbst, A., Ljungh, A., Svenningsen, N., Hagerstrand, I. and Nystrom, L. (2001) Intrauterine infection may be a major cause of stillbirth in Sweden. *Acta Obstet Gynecol Scand*, **80**, 511-518.

Toth, A. and Lesser, M. L. (1981) Asymptomatic bacteriospermia in fertile and infertile men. *Fertil Steril*, **36**, 88-91.

Toth, A., O'Leary, W. M. and Ledger, W. (1982) Evidence for microbial transfer by spermatozoa. *Obstet Gynecol*, **59**, 556-559.

Toth, B., Jeschke, U., Rogenhofer, N., Scholz, C., Wurfel, W., Thaler, C. J. and Makrigiannakis, A. (2010) Recurrent miscarriage: Current concepts in diagnosis and treatment. *J Reprod Immunol*, **85**, 25-32.

Toth, F. D., Aboagye-Mathiesen, G., Nemes, J., Liu, X., Andirko, I., Hager, H., Zdravkovic, M., Szabo, J., Kiss, J., Aranyosi, J. and Ebbesen, P. (1997) Epstein-barr virus permissively infects human syncytiotrophoblasts *in vitro* and induces replication of human T cell leukemia-lymphoma virus type I in dually infected cells. *Virology*, **229**, 400-414.

Toth, I. E., Wiesel, O., Boldogkoi, Z., Balint, K., Tapaszti, Z. and Gerendai, I. (2007) Predominance of supraspinal innervation of the left ovary. *Microsc Res Tech*, **70**, 710-718.

Tureck, R., Garcia, C., Blasco, L., Mastroianni, L. (1993) Perioperative complications arising after trans-vaginal oocyte retrieval. *Obstet Gynecol*, **81**, 590-593.

Upadhyaya, M., Hibbard, B. M. and Walker, S. M. (1984) The effect of *Ureaplasma urealyticum* on semen characteristics. *Fertil Steril*, **41**, 304-308.

Urbina, S., Ruiz, H., Parejas, S. (2006) Pelvic actinomyces infection: Report of two cases occurred in the hospital of San Jose. *Infect Dis Obstet Gynecol*, 1-2.

Van Blerkom, J., Antczak, M. and Schrader, R. (1997) The developmental potential of the human oocyte is related to the dissolved oxygen content of follicular fluid: Association with vascular endothelial growth factor levels and perifollicular blood flow characteristics. *Hum Reprod*, **12**, 1047-1055.

van Geertruyden, J., Thomas, F., Erhart, A., D'Alessandro, U. (2004) The contribution of malaria in pregnancy to perinatal mortality. *Am J Trop Med Hyg*, **7**, 93-104.

Venegas, M. F., Navas, E. L., Gaffney, R. A., Duncan, J. L., Anderson, B. E. and Schaeffer, A. J. (1995) Binding of type 1-piliated *Escherichia coli* to vaginal mucus. *Infect Immun*, **63**, 416-422.

Vercellini, P., Aimi, G., De Giorgi, O., Maddalena, S., Carinelli, S. and Crosignani, P.G. (1998) Is cystic ovarian endometriosis an asymmetric disease? *Br J Obstet Gynaecol*, **105**, 1018-21.

Verhelst, R., Verstraelen, H., Claeys, G., Verschraegen, G., Van Simaey, L., De Ganck, C., De Backer, E., Temmerman, M. and Vaneechoutte, M. (2005) Comparison between Gram-stain and culture for the characterisation of vaginal microflora: Definition of a distinct grade that resembles grade I microflora and revised categorisation of grade I microflora. *BMC Microbiol*, **5**, 61-71.

Vielfort, K., Sjolinder, H., Roos, S., Jonsson, H. and Aro, H. (2008) Adherence of clinically isolated lactobacilli to human cervical cells in competition with *Neisseria gonorrhoeae*. *Microbes Infect*, **10**, 1325-1334.

Vigil, P., Morales, P., Tapia, A., Riquelme, R. and Salgado, A. M. (2002a) *Chlamydia trachomatis* infection in male partners of infertile couples: Incidence and sperm function. *Andrologia*, **34**, 155-161.

Vigil, P., Tapia, A., Zacharias, S., Riquelme, R., Salgado, A. M. and Varleta, J. (2002b) First-trimester pregnancy loss and active *Chlamydia trachomatis* infection: Correlation and ultrastructural evidence. *Andrologia*, **34**, 373-378.

Villegas, J., Schulz, M., Soto, L., Iglesias, T., Miska, W. and Sanchez, R. (2005a) Influence of reactive oxygen species produced by activated leukocytes at the level of apoptosis in mature human spermatozoa. *Fertil Steril*, **83**, 808-810.

Villegas, J., Schulz, M., Soto, L. and Sanchez, R. (2005b) Bacteria induce expression of apoptosis in human spermatozoa. *Apoptosis*, **10**, 105-110.

Villegas, L., McGready, R., Htway, M., Paw, M. K., Pimanpanarak, M., Arunjerdja, R., Viladpai-Nguen, S. J., Greenwood, B., White, N. J. and Nosten, F. (2007) Chloroquine prophylaxis against vivax malaria in pregnancy: A randomized, double-blind, placebo-controlled trial. *Trop Med Int Health*, **12**, 209-218.

Viniker, D. A. (1999) Hypothesis on the role of sub-clinical bacteria of the endometrium (bacteria endometrialis) in gynaecological and obstetric enigmas. *Hum Reprod Update*, **5**, 373-385.

Virecoulon, F., Wallet, F., Fruchart-Flamenbaum, A., Rigot, J. M., Peers, M. C., Mitchell, V. and Courcol, R. J. (2005) Bacterial flora of the low male genital tract in patients consulting for infertility. *Andrologia*, **37**, 160-165.

Vossen, R. H., Aten, E., Roos, A. and den Dunnen, J. T. (2009) High-resolution melting analysis (HRMa): More than just sequence variant screening. *Hum Mutat*, **30**, 860-866.

Vujisic, S., Lepej, S. Z., Emedi, I., Bauman, R., Remenar, A. and Tiljak, M. K. (2006) Ovarian follicular concentration of IL-12, IL-15, IL-18 and p40 subunit of IL-12 and IL-23. *Hum Reprod*, **21**, 2650-2655.

Walsh, C., Mayer, E., Baxi, L. (2006) Lyme disease in pregnancy: Case report and review of the literature. *Obstet Gynecol Survey*, **62**, 41-50.

Wang, M. W., Sims, M. J., Symington, P. R., Humphreys, A. S. and Taussig, M. J. (1991) Induction of anti-progesterone immunity and pregnancy blocking by anti-progesterone anti-idiotypes. Variable efficacy of polyclonal Ab2 antibodies directed against a panel of closely related Ab1 antibodies. *Immunology*, **73**, 348-355.

Wang, T. H., Chang, C. L., Wu, H. M., Chiu, Y. M., Chen, C. K. and Wang, H. S. (2006) Insulin-like growth factor-II (IGFII), IGF-binding protein-3 (IGFBP-3), and -BP4 in follicular fluid are associated with oocyte maturation and embryo development. *Fertil Steril*, **86**, 1392-1401.

Wang, Y. A., Chambers, G.M., Dieng, M. and Sullivan, E.A. (2009) Assisted reproductive technology in Australia and New Zealand 2007. Assisted reproduction technology series no. 13. In AIHW (ed). AIHW., Canberra.

Wang, Y. A., Chambers, G.M., Dieng, M. and Sullivan, E.A. (2010) Assisted reproductive technology in Australia and New Zealand 2008. Assisted reproduction technology series no. 14. In AIHW (ed). AIHW., Canberra.

Watts, D. H., Krohn, M. A., Hillier, S. L. and Eschenbach, D. A. (1990) Bacterial vaginosis as a risk factor for post-caesarean endometritis. *Obstet Gynecol*, **75**, 52-58.

Weidner, W., Schiefer, H. G. and Garbe, C. (1987) Acute nongonococcal epididymitis. Aetiological and therapeutic aspects. *Drugs*, **34**, 111-117.

Weidner, W., Krause, W. and Ludwig, M. (1999) Relevance of male accessory gland infection for subsequent fertility with special focus on prostatitis. *Hum Reprod Update*, **5**, 421-432.

Wessler, S., Muenzner, P., Meyer, T.F. and Naumann, M. (2005) The anti-inflammatory compound curcumin inhibits *Neisseria gonorrhoeae*-induced NF-kappa β signalling, release of pro-inflammatory cytokines/chemokines and attenuates adhesion in late infection. *Biol Chem*, **386**, 481-90.

Westrom, L. and Eschenbach, D. (1999) Pelvic inflammatory disease. In Holmes, K. K. *et al*. (eds.) *Sexually Transmitted Diseases*. McGraw-Hill, New York.

Wiesenfeld, H. C., Hillier, S. L., Krohn, M. A., Amortegui, A. J., Heine, R. P., Landers, D. V. and Sweet, R. L. (2002) Lower genital tract infection and endometritis: Insight into subclinical pelvic inflammatory disease. *Obstet Gynecol*, **100**, 456-463.

Wikland, M., Enk, L., Hammarberg, K. and Nilsson, L. (1987) Use of a vaginal transducer for oocyte retrieval in an IVF/ET program. *J Clin Ultrasound*, **15**, 245-251.

Wilson, J. D., Ralph, S. G. and Rutherford, A. J. (2002) Rates of bacterial vaginosis in women undergoing *in vitro* fertilisation for different types of infertility. *Brit J Obstet Gynaecol*, **109**, 714-717.

Wilson, M. (2005) *Microbial Inhabitants of Humans : Their ecology and role in health and disease*. Cambridge University Press, Cambridge.

Wilson, R., Jenkins, C., Miller, H., McInnes, I. B., Moore, J., McLean, M. A. and Walker, J. J. (2004 a) Abnormal cytokine levels in non-pregnant women with a history of recurrent miscarriage. *Eur J Obstet Gynecol Reprod Biol*, **115**, 51-54.

Wilson, R., Moor, J., Jenkins, C., Miller, H., Walker, J. J., McLean, M. A., Norman, J. and McInnes, I. B. (2004 b) Abnormal first trimester serum interleukin-18 levels are associated with a poor outcome in women with a history of recurrent miscarriage. *Am J Reprod Immunol*, **51**, 156-159.

Winberg, J., Gezelius, L., Guldevall, L. and Mollby, R. (1993) Cephadroxil promotes vaginal colonisation with *Escherichia coli*. *Infection*, **21**, 201-205.

Wintermute, E. H. and Silver, P. A. (2010 a) Dynamics in the mixed microbial concourse. *Genes Dev*, **24**, 2603-2614.

Wintermute, E. H. and Silver, P. A. (2010 b) Emergent cooperation in microbial metabolism. *Mol Syst Biol*, **6**, 407-413.

Witkin, S., Linhares, I., Giraldo, P. (2007) Bacterial flora of the female genital tract: Function and immune regulation. *Best Pract Res Clin Obstet Gynecol*, 21(3):347-54.

Witkin, S. S. (1999) Immunity to heat shock proteins and pregnancy outcome. *Infect Dis Obstet Gynecol*, **7**, 35-38.

Wolff, H., Neubert, U., Zebhauser, M., Bezold, G., Korting, H. C. and Meurer, M. (1991) *Chlamydia trachomatis* induces an inflammatory response in the male genital tract and is associated with altered semen quality. *Fertil Steril*, **55**, 1017-1019.

Wolner-Hanssen, P. and Mardh, P. A. (1984) *In vitro* tests of the adherence of *Chlamydia trachomatis* to human spermatozoa. *Fertil Steril*, **42**, 102-107.

Wolner-Hanssen, P., Kiviat, N., Holmes, K. (1990) Atypical pelvic inflammatory disease: Subacute, chronic, or subclinical upper genital tract infection in women. In Holmes, K. K. *et al*. (eds.) *Sexually Transmitted Diseases*. McGraw-Hill, New York.

Woolley, P. D. (2000) Anaerobic bacteria and non-gonococcal urethritis. *Int J STD & AIDS*, **11**, 347-348.

World Health Organisation. (1999) *Who laboratory manual for the examination of human semen and sperm-cervical mucus interaction*. 4th edn, Cambridge University Press, Cambridge.

World Health Organisation. (2006) *Global Strategy for the Prevention and Control of Sexually Transmitted Infections: 2006–2015*. Cambridge University Press, Cambridge.

Wu, M. Y. and Ho, H. N. (2003) The role of cytokines in endometriosis. *Am J Reprod Immunol*, **49**, 285-296.

Wu, R., Van der Hoek, K. H., Ryan, N. K., Norman, R. J. and Robker, R. L. (2004) Macrophage contributions to ovarian function. *Hum Reprod Update*, **10**, 119-133.

Xiao, L., Glass, J. I., Paralanov, V., Yooseph, S., Cassell, G. H., Duffy, L. B. and Waites, K. B. (2010) Detection and characterisation of human Ureaplasma species and serovars by real-time PCR. *J Clin Microbiol*, **48**, 2715-2723.

Xu, M., Morishima, N., Mizoguchi, I., Chiba, Y., Fujita, K., Kuroda, M., Iwakura, Y., Cua, D. J., Yasutomo, K., Mizuguchi, J. and Yoshimoto, T. (2011) Regulation of the development of acute hepatitis by IL-23 through IL-22 and IL-17 production. *Eur J Immunol*, E print Jul18.

Yin, L. R., Sun, J. J., Ma, H. D., Mi, S. L., Guo, S. J. and Shi, Y. (2006) [Expression of interleukin1-α, β and interferon-γ in macrophages from endometrium of women with endometriosis]. *Zhonghua Fu Chan Ke Za Zhi*, **41**, 295-298. Abstract only

Yun, T. H., Morrissey, J. H. (2009) Polyphosphate and omptins: novel bacterial pro-coagulant agents. *J Cell Mol Med*, **13**, 4146-4153.

Zamboni, L., Hongsanand, C. and Mastroianni, L., Jr. (1965) Influence of tubal secretions on rabbit tubal ova. *Fertil Steril*, **16**, 177-184.

Zegans, M. E., Becker, H. I., Budzik, J. and O'Toole, G. (2002) The role of bacterial biofilms in ocular infections. *DNA Cell Biol*, **21**, 415-420.

Zegers-Hochschild, F., Adamson, G. D., de Mouzon, J., Ishihara, O., Mansour, R., Nygren, K., Sullivan, E., van der Poel, S., on behalf of, ICMART and WHO (2009) The international committee for monitoring assisted reproductive technology (ICMART) and the world health organisation (WHO) revised glossary on ART terminology, 2009. *Hum Reprod*, **24**, 2683-2687.

Zur, K. B., Mandell, D. L., Gordon, R. E., Holzman, I. and Rothschild, M. A. (2004) Electron microscopic analysis of biofilm on endotracheal tubes removed from intubated neonates. *Otolaryngol Head Neck Surg*, **130**, 407-414.