

# The Isolation and Identification of Microorganisms in the Reproductive Environment: The Potential Impact on the IVF Culture System and on IVF Outcomes

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**I**n vitro fertilization (IVF) treatment outcomes have continued to improve with the implementation of progressive techniques including the introduction of sequential culture media, blastocyst culture, assisted hatching and pre-implantation screening; however, despite overcoming the barriers to conception, and the precise control over so many pre and post conception aspects, the global IVF success rates remain reasonably static (Beck *et al.*, 2010, Das *et al.*, 2009, Gardner and Lane, 1997, Geary, 2006, Handyside *et al.*, 1992, Munne *et al.*, 1993, Palermo *et al.*, 1992). Preterm birth also continues to be an increasing global problem, which exists in IVF even with the best practice shift from multiple embryo transfers to single embryo transfers, resulting in a reduction in multiple gestations (Beck *et al.*, 2010, Vulliemoz, 2012). The presence of microorganisms within the upper genital tract, and those that contaminate the IVF culture system may result in poor quality oocytes, poor quality embryos (possibly due to oocyte DNA fragmentation), early pregnancy loss or preterm birth.

IVF procedures offer a unique opportunity for screening biological fluids (ovarian follicular fluid, Fallopian tube washings, peritoneal fluid and endometrial aspirates) that would not otherwise be available due to the invasive nature of the collection procedure. A small number of researchers have reported isolating microorganisms from the IVF culture system, with mixed reports surrounding the significance of such a discovery (Artley *et al.*, 1993,

Cottell *et al.*, 1996, Pelzer *et al.*, 2011). The microbiota (microorganisms, their genetic elements and the environment in which they are living) at each

of these sites has the potential to be transferred to additional sites by iatrogenic procedures involved in the IVF cycle (Table I).

## Do we really believe that the female upper genital tract is sterile?

An increasing body of evidence suggests that the female upper genital tract is not as sterile as once thought (Spence *et al.*, 1982, Viniker, 1999, Pelzer *et al.* unpublished data). Notably, clinical predictors traditionally used for diagnosing an overt infection (febrile temperatures, purulent discharge, pain, raised white cell counts), are absent even in the presence of a diverse range of microorganisms, suggesting that the female upper genital tract is at the very least transiently colonized in healthy women. Importantly, asymptomatic inflammation of the endometrial cavity results in decreased conception rates for both natural and IVF cycles (Feghali *et al.*, 2003, Taylor and Frydman, 1996). Is knowledge of the upper genital tract microbiome relevant for assisted reproduction technology? Can we use our new knowledge of the upper genital tract microbiome to explain shortcomings in IVF, particularly in couples with idiopathic infertility? Can we improve IVF outcomes using appropriate, timed, currently available antimicrobial therapy?

## What is the anatomical source of the follicular fluid microorganisms?

A diverse range of microorganisms have been detected in human follicular fluids collected from women at the time of trans-vaginal oocyte retrieval, from laparoscopically collected peritoneal fluid at the time of tubal embryo transfer (Artley *et al.*, 1993,

Cottell *et al.*, 1996, Pelzer *et al.*, 2011, Saltes, 1995). There remains some controversy around whether these microorganisms represent colonization of the upper genital tract or merely contamination of the ovarian follicular fluid by the vaginal flora at the time of puncture for trans-vaginal oocyte aspiration (Cottell *et al.*, 1996, Pelzer *et al.*, 2011). We concluded from our data that microorganisms could be present in either a concordant or a discordant fashion, similar to that reported by Spence *et al.* (1982) in their study of paired cervical and endometrial samples. In our study, microorganisms were defined as concordant if present in both the upper genital tract (follicular fluids) and in the lower genital tract (vaginal secretions) samples collected from the same woman. A discordant microbiota was

defined as a difference in the microorganisms detected at each of these sites, suggesting that the upper and the lower genital tract were independently colonized by microorganisms. We propose that the discordant follicular fluid isolates possibly represent an extension of the normal regional flora of the lower genital tract and transient flora of the endometrium, resulting in a specific colonization pattern within the follicular fluid. Further, these species may also represent a continuum from the lower genital tract to the upper genital tract. Finally, the microorganisms detected in both the follicular fluids and the vaginal secretions from the same woman may merely represent contaminating species transferred into the follicular fluid during the oocyte retrieval process.

*Table I. Sources of microbial contamination of the IVF culture system and their potential impact, prevention and treatment*

Site of IVF contamination	Source of microorganisms in IVF					Potential IVF impact	Prevention	Treatment
	Female partner				Male partner			
	Vagina	Endometrium	Fallopian tubes	Follicular fluid	Semen/ spermatozoa			
TVOR needle	•			•		Contamination of the IVF culture system	Change microbiota	
Culture media	•			•	•	Contamination of gametes and embryos by microbes and/or microbial metabolites	Antimicrobials included in culture media	Alter antimicrobials
Gametes	•			•	•	Cell damage ± apoptosis caused by microbes or microbial metabolites	Inhibit growth of pathogens in culture media	
Embryo transfer catheter		•		•	•	Inoculation of the endometrium	Inhibit growth of pathogens in culture media	
Embryos		•		•	•	Developmental arrest	Inhibit growth of pathogens in culture media	
Endometrium			•	•	•	Endometritis		Antimicrobial treatment
Fallopian tubes		•		•		Retrograde infection via the endometrium		Antimicrobial treatment
Peritoneal cavity		•	•	•		Retrograde infection via the endometrium and Fallopian tubes		Antimicrobial treatment

## IVF outcomes and follicular fluid microorganisms

Data from our recent study suggest that the source of the microorganisms is less important than the fact that they are present in the follicular fluid in the first instance (Pelzer *et al.*, 2011). In our study, there was no relationship between follicular fluid colonization or contamination and the cause of infertility. However, the microbial contamination of the IVF culture system was associated with an increase in the number of embryo discards, a reduced embryo transfer rate and a reduced pregnancy rate if women had a history of endometriosis. We reported the same results for women who had an infertile male partner, perhaps suggesting a compounding role for the abnormal inflammatory response documented in the reproductive tract of these women, rather than a direct effect from the microorganisms (Robertson *et al.*, 2002, Weiss *et al.*, 2009). An earlier study reported a similar finding of poor IVF outcomes for women with endometriosis and positive IVF cultures but not for women with ovulatory dysfunction or infertile male partners (Saltes *et al.*, 1995). Differences in the conclusions of these studies may reflect the differing sample sizes.

Microbes may contribute to the inflammatory environment and the IVF process may further serve to reactivate 'infections' in these women or to transfer microorganisms from one anatomical site to another as previously reported (Baber *et al.*, 1988, Howe *et al.*, 1988, Yuzpe *et al.*, 1989). Despite the proposed source of the microorganisms, follicular fluid microorganisms were associated with both positive and negative IVF outcomes (Pelzer *et al.*, 2011). This is consistent with prior reports for bacterial vaginosis, endometritis and adverse pregnancy outcomes.

When *Propionibacterium* spp., *Streptococcus* spp., *Actinomyces* spp., *Staphylococcus* spp., *Bacteroides* spp. and *Bifidobacterium* spp. were isolated from follicular fluids, we observed an increase in adverse IVF outcomes including decreased fertilization rates, decreased embryo transfer rates, decreased pregnancy rates or no pregnancy (failed implantation) which may reflect poor quality embryos due to damage by the microorganisms themselves, microbial metabolites, or a hostile implantation environment. The presence of high numbers of *Lactobacillus* spp. within follicular fluid

frequently correlated with positive IVF outcomes. We also observed differences between the microorganisms isolated from the left and the right ovarian follicular fluids and in the corresponding IVF outcomes for each side. Women diagnosed with ovarian endometriomas display a similar trend, suggesting that the separate vasculature and anatomy within each side of the pelvis is important in pathology (Al-Fozan and Tulandi, 2003, Last, 1984, Sznurkowski and Emerich, 2008, Vercellini *et al.*, 1998). We propose that the slower drainage of the left ovary might promote the persistence of microorganisms at that site.

## Persistent growth patterns of follicular fluid microorganisms

Many genital tract isolates are capable of forming biofilms. The lactobacilli are particularly successful in this regard and the metabolic products that they produce ( $H_2O_2$ , lactic acid, peroxidase) create an environment that does not favour colonization by other species in high numbers (Klebanoff and Coombs, 1991, Klebanoff *et al.*, 1991, Martin *et al.*, 1999). However, the lactobacilli co-exist with a diverse range of microbial species in the genital tract.

Microorganisms traditionally live either as free-floating planktonic cells or as communities within biofilms. By growing as biofilms, they become metabolically inactive and develop a protective outer coating composed of polysaccharide, nucleic acid, mineral and protein capable of inhibiting detection and management by the host immune response, antimicrobial treatment and chemical disinfection (Trinidad, 2010). We studied the long-term *in vitro* growth of microorganisms isolated from human follicular fluid and report that they persist for at least 28 weeks without the nutrient supplementation or waste removal, which would occur *in vivo*. Other studies have similarly examined the persistence of *Gardnerella vaginalis*, *Neisseria gonorrhoeae* and lactobacilli on genital tract cells. Some species of lactobacilli are capable of disrupting *Gardnerella* spp. biofilm surface area, density and depth (Saunders *et al.*, 2007). *Gardnerella* spp. associated with bacterial vaginosis reportedly resists standard treatment with antimicrobial agents, promoting the recurrence of infection in subsequent menstrual cycles (Swidsinski *et al.*, 2005).

Interestingly, pure cultures of *Gardnerella vaginalis* do not always cause bacterial vaginosis, and low numbers of this species frequently colonize the female lower genital tract (Patterson *et al.*, 2009, Srinivasan *et al.*, 2010). In some women, the lower genital microbiota frequently reverts to bacterial vaginosis during menses, despite repeated treatment and apparent resolution of the condition (Srinivasan *et al.*, 2010). Similar to their interaction with *Gardnerella* spp., some lactobacilli also compete with gonococci for attachment to genital tract epithelial cells and are capable of reducing gonococcal invasion of the epithelial cells (Spurbeck and Arvidson, 2008, Vielfort *et al.*, 2008). IVF studies have reported that the genital tract lactobacilli are stable once estradiol levels become elevated (Jakobsson and Forsum, 2008). The same species have also been reported as dominant isolates in the vaginal flora of women of reproductive age (Ravel *et al.*, 2011). Recent studies confirm that the vaginal microbiota changes throughout the menstrual cycle because of steroid hormone levels (Hyman *et al.*, 2012, Jakobsson and Forsum, 2008, Srinivasan *et al.*, 2010). Some women exhibit a healthy vaginal microbiota, which is composed predominantly of species thought to be causal in bacterial vaginosis. Such women achieved pregnancies but no data are available to assess the impact of these species on a developing pregnancy (Ravel *et al.*, 2011).

Biofilms are associated with any environment where there is a liquid-solid interface. The biofilms may also represent a protected form of growth, which can exist in a mutualistic relationship (for example with the vaginal lactobacilli) when formed within the correct site. However, once disrupted, as is the case in trans-vaginal oocyte retrieval and possibly to a lesser extent, during embryo transfer, then the dormant biofilm cells have the potential to translocate to other genital tract sites. Once again metabolically active, the microbial cells may replicate and cause contamination or overt infection, or elicit an inflammatory response by antigenic properties of the individual cells or their products of metabolism (Kaplan and Fine, 2002, Kaplan, 2010).

There is the potential to alter the genital tract microbiota using probiotics and knowledge of the hormonal milieu during the menstrual cycle. It is in theory possible to repopulate the genital tract with 'good' endogenous species (Mailander-Sanchez *et al.*,

2012). There is evidence to suggest that rectal isolates can result in opportunistic infection of the genital tract and adverse pregnancy outcomes, and the use of gastrointestinal tract normal regional flora as probiotics in women undergoing IVF treatment has not proved useful in improving IVF outcomes (Gilboa *et al.*, 2005). However, studies investigating the protective role of lactobacilli in reducing gonococcal infection support the notion that to have a beneficial effect, the species must originate from the endogenous vaginal lactobacilli (Vielfort *et al.*, 2008).

### Antimicrobial prophylaxis

Antimicrobial prophylaxis has attracted research in IVF as an important consideration prior to performing any invasive procedures. In our IVF unit, we screen couples for the blood borne viruses, Hepatitis B and C, and HIV prior to entry into an IVF cycle as per the National guidelines for the collection and storage of human tissue. In addition, we also screen (and treat) both partners for genital tract infections. In our study to assess whether the cause of infertility or underlying pathology was related to variations in the follicular fluid or vaginal microbiota, antimicrobial treatment prior to trans-vaginal oocyte retrieval or following embryo transfer was not required for any women. A single dose of a second-generation cephalosporin was routinely administered after anaesthetic induction for trans-vaginal oocyte retrieval.

Of note, the majority of bacterial species identified within follicular fluids were anaerobes. We highlight that these are generally not targeted by penicillin, streptomycin or gentamicin, the antimicrobials routinely included in IVF culture media, or the cephalosporins used for prophylaxis in gynaecological surgery (non-hysterectomy, non-termination of pregnancy). Antimicrobial treatment in our IVF clinic was based on those prescribed in the Therapeutic guidelines (antibiotic), a National reference tool for pharmaceutical prescribing in Australia. Antimicrobial sensitivity studies were performed using the CDS method (Bell, 2012, Antibiotic Expert Group, 2010). We note that this regimen may be different in other countries.

Future studies should aim to establish the potential for antimicrobial embryotoxicity as well as the minimum inhibitory concentrations of alternative antimicrobials



that could be added to the IVF culture media. Curtis *et al.* (1991) suggested that the antibiotics in culture media might not actually reach a therapeutic range. We propose that couples who have repeated IVF failures and whose samples (follicular fluids, vaginal swabs, washed semen, IVF culture media) have been screened for the presence of, and shown to harbour microorganisms resistant to those routinely employed in the IVF culture system and treatment cycle, might benefit from such a change.

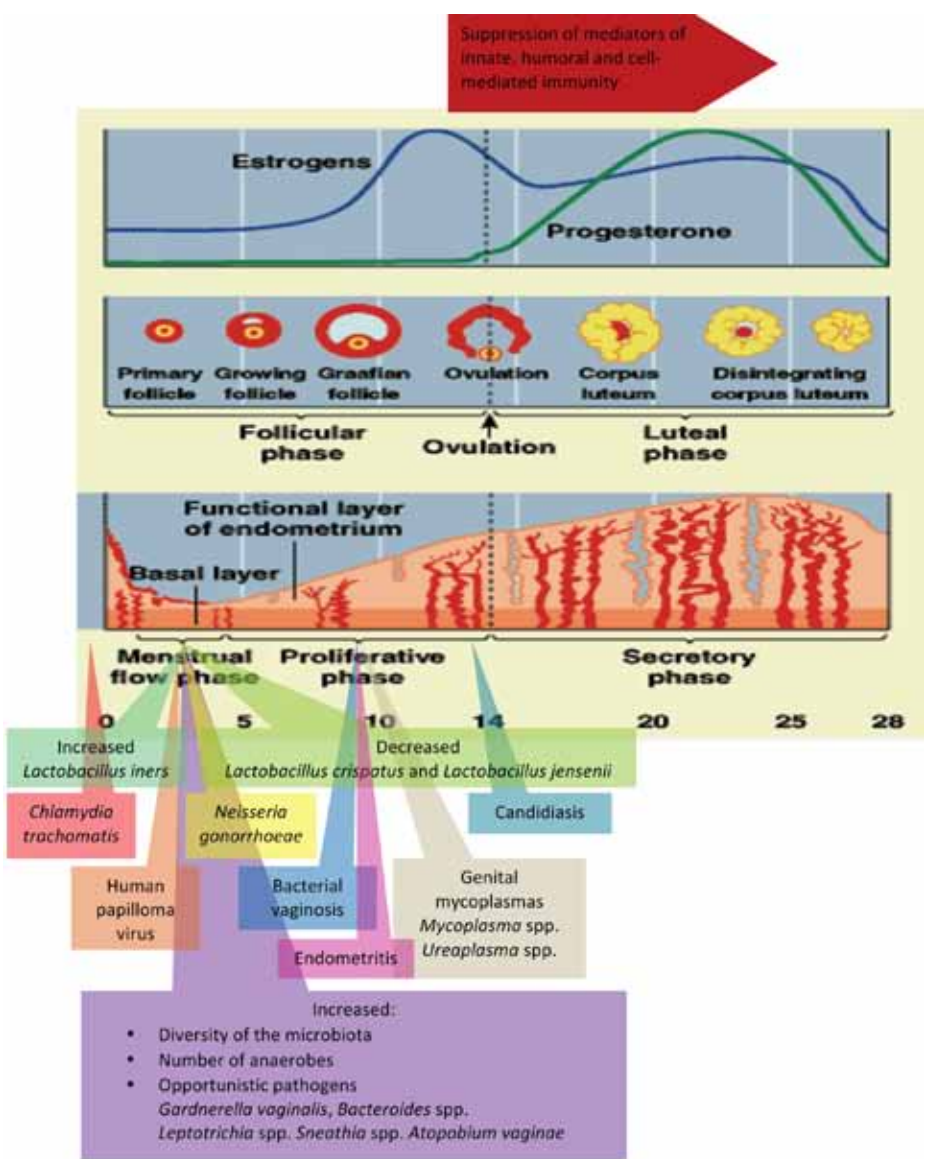
The aim throughout the IVF cycle should be to promote the growth of a normal regional flora within the genital tract and the gastrointestinal tract. Antimicrobial therapy during an IVF cycle may actually be detrimental to the IVF outcomes by altering the normal microbiota in the genital tract (Huyser *et al.*, 1991, Liversedge *et al.*, 1996). Studies investigating the treatment of both partners concluded that there was no advantage of treatment in the absence of infection; rather the results were likely to be detrimental to the cycle outcomes (Gallegos *et al.*, 2008, Liversedge *et al.*, 1996).

### Hormone dependent microbial growth patterns and the menstrual cycle

A close relationship exists between the regulation of microbial growth in the female genital tract and the menstrual cycle. Reports of genital tract infections occur in a cycle-dependent fashion (Reviewed by Sonnex, 1998), attributed to changes in the secretion levels of the steroid hormones estradiol and progesterone and the ability of some microbial species to use these

Figure 1.

Just prior to ovulation, dampening down of all aspects of the female the immune response occurs in preparation for genital tract priming by the seminal fluid, followed by fertilization, implantation and pregnancy. The relationship between the menstrual cycle steroid hormones, estradiol and progesterone, and genital tract infection diagnoses is illustrated. The menstrual and proliferative phases of the menstrual cycle demonstrate the greatest alterations in the genital tract microbiota. Thus, diagnosis of the majority of female genital tract infections also occurs in the first half of the cycle.



hormones as growth factors (Kornman and Loesche, 1982). The *Lactobacillus* spp. concentration is highest when estradiol levels are high, and the concentration of opportunistic pathogens reportedly increases during menstruation (Figure 1). Subsequent changes in the composition of the genital tract epithelium, the secretion of mucous and the localized immune response, follow the pattern of the hormonal changes (Brabin, 2001, Ceruti *et al.*, 1994, McGregor *et al.*, 1990, Sonnex, 1998). In women undergoing IVF cycles, the hormone levels of the steroid hormones estradiol and progesterone are reportedly eight and three fold higher respectively than in normal cycling women (Kushnir *et al.*, 2009).

The steroid hormones also contribute to the physiological function of the genital tract. For example, the vaginal epithelial thickness, parakeratosis and glycogen content rise at mid-cycle (Nauth and Haas, 1985). The cervico-vaginal secretions become thicker, clearer and more elastic around the time of ovulation (Farage and Maibach, 2006). The endometrium undergoes significant remodelling throughout the cycle with secretory cells swelling to produce cytoplasmic projections and changes in the microvilli and an increase in secretory granules in the ovulatory stage (Hayashidani and Fujiwara, 1986). In the Fallopian tube, estradiol stimulates epithelial cell hypertrophy, secretion and ciliogenesis, whilst high levels of progesterone promote atrophy and deciliation (Donnez *et al.*, 1985, Verhage *et al.*, 1979). Ciliary beat frequency also changes throughout the menstrual cycle (Lyons, 2006). Remodelling of the ovary occurs throughout the cycle to produce the dominant follicle and then the corpus luteum. It is therefore likely that the microbial growth patterns, and the changes in the genital tract epithelium and secretions that occur in response to the secretion of the menstrual cycle steroid hormones, combine to determine the microbiota detected at each genital tract site. It would be interesting to examine changes in the genital tract microbiota of women using luteal support following embryo transfer to determine the impact of the hormonal changes observed in natural cycles, fully stimulated cycles and hormone replacement therapy cycles.

In addition to the ovarian follicular fluid aspirates, microorganisms can also be recovered from the peritoneal fluids of women undergoing assisted

reproduction, from Fallopian tube washings following falloposcopy, from the endometrium of women with and without a history of bacterial vaginosis and from pre-term placentas delivered vaginally or by Caesarean section (Cottell *et al.*, 1996, DiGiulio *et al.*, 2010a, DiGiulio *et al.*, 2010b, Korn *et al.*, 1995, Onderdonk *et al.*, 2008a, Onderdonk *et al.*, 2008b, Pelzer *et al.*, 2011, Saltes, 1995). The lactobacilli are among those species frequently identified from each of these upper genital tract sites. Lactobacilli are often difficult to isolate but can be recovered from clinical samples in the presence and absence of upper genital tract infection and in placentas delivered at term or preterm (Andrews *et al.*, 2005, Debattista *et al.*, 2004, DiGiulio *et al.*, 2010a, DiGiulio *et al.*, 2010b, Korn *et al.*, 1995, Onderdonk *et al.*, 2008a, Onderdonk *et al.*, 2008b, Srinivasan *et al.*, 2010). Changes occur in the lactobacillus concentration in response to antibiotic treatment, but remain reasonably constant throughout the cycle in normal cycling women and in those undergoing ovarian stimulation cycles for IVF (Jakobsson and Forsum, 2008, Srinivasan *et al.*, 2010). Therefore, fluctuations in the numbers of opportunistic pathogens comprising the microbiota at these sites may be a more relevant indicator for predicting adverse outcomes including poor fertilization, poor embryo quality, failed implantation and early pregnancy loss.

### The immune response and reproductive health

Several studies have reported correlations between cytokines and IVF outcomes. Indeed, it would appear that some might serve as useful predictors of IVF outcomes and even the cause of infertility (Fasciani *et al.*, 2000, Van Blerkom *et al.*, 1997). What is apparent from the literature is that an accurate determination of the concentration of the genital tract cytokines cannot occur by measuring those in the systemic circulation. In addition to the systemic cues, the genital tract cytokines are synthesised within each tissue in response to local stimuli (Dunbar *et al.*, 2012, Ochiel *et al.*, 2008, Wira *et al.*, 2005). It would appear that the immune response skew to either pro-inflammatory or anti-inflammatory at the correct time in the cycle is essential to ensure that each process (menstruation, ovulation, fertilization, implantation, placentation and ongoing pregnancy) succeeds.

In our study, the cytokine responses were

independent of the microbiota isolated from both the follicular fluids and the vaginal secretions using direct culture techniques. That is, we failed to identify any correlation between the cytokines reported within the follicular fluids or vaginal secretions from women with both concordant or discordant upper and lower genital tract microbiota (Pelzer *et al.*, 2011). Therefore, we suggest that the microbial isolates in ovarian follicular fluids do not represent an overt infection but rather normal regional flora or contamination.

Spandorfer *et al.* (2001) reported alterations in the pro-inflammatory cytokines detected in the cervical secretions in women with abnormal vaginal flora, but they did not report a correlation between the altered cytokine response and the IVF outcomes. This further supports the localized role of the immune mediators. In our study, we did correlate the cytokine expression with the cause of infertility and the IVF outcomes for couples with idiopathic infertility. It is now also apparent that subtle changes in the genes that regulate the immune response can influence the infection related sequelae (Genc and Onderdonk, 2011, Witkin *et al.*, 2000). Site-specific screening of immune markers may be useful in couples with repeated IVF failures. In the future, we may also see a shift towards genetic screening in such couples.

### Screening recommendations to identify genital tract pathogens

Based on our current knowledge, screening for reproductive infections should occur at the correct stage of the menstrual cycle in the female partner to maximise isolation of the microorganism. It would be interesting to examine the microbiota in women with repeated IVF failures as a function of the type of ovarian stimulation cycle, to gain an understanding of the changes in the microbiota that might be induced by differences in the hormonal profile. Our samples were collected from women undergoing ‘boost’ cycles without antibiotic cover and so the microbiota may well be different from that observed in other clinics with alternative hormonal stimulation protocols as well as methods for vaginal preparation prior to trans-vaginal oocyte retrieval and antibiotic coverage prior to oocyte retrieval and throughout the treatment cycle. Screening of the vaginal and/or gastrointestinal microbiota may offer an opportunity for non-invasive treatment of infertile women or women with a history of repeated spontaneous abortion prior to commencement of IVF.

### Recommendations and conclusions in the IVF setting

Metagenomic analyses performed as part of the human microbiome project have shed light on the vast

Table II. Recommendations for reducing the impact of microorganisms on the IVF culture system

<b>Changes to culture techniques</b>	<ul style="list-style-type: none"> <li>➤ Add rapid media changes to dilute microorganisms out of culture media</li> <li>➤ Freeze all embryos in couples with repeated IVF failures and contaminated samples to allow antimicrobial treatment of the female partner prior to embryo transfer</li> <li>➤ Perform antimicrobial sensitivity assays on pathogens isolated from clinical samples</li> <li>➤ Determine minimum inhibitory concentrations for antibiotics in culture media</li> <li>➤ Identify alternate antimicrobials and assess embryotoxicity</li> </ul>
<b>Screening of patient samples</b>	<ul style="list-style-type: none"> <li>➤ Determine the most appropriate samples to screen</li> <li>➤ Determine the microbiota present in each sample</li> <li>➤ Determine the immune system response by measuring cytokines and chemokines in clinical samples</li> <li>➤ Probiotic treatment to restore normal genital tract microbiota</li> <li>➤ Antimicrobial treatment for couples prior to oocyte retrieval or embryo transfer if repeated failed cycles and microorganisms consistently isolated from samples</li> </ul>
<b>Further research</b>	<ul style="list-style-type: none"> <li>➤ Determine the impact of media changes and antimicrobials on gametes and embryos</li> <li>➤ Identify and characterize the microbial species that are pathogenic at each stage of IVF</li> <li>➤ Determine the role of the host immune response in adverse outcomes where pathogens are identified</li> </ul>



differences in the vaginal microbiota (Hyman *et al.*, 2012, Ravel *et al.*, 2011, Srinivasan *et al.*, 2010). It is likely that the impact of microorganisms on IVF will need to be assessed on a case-by-case basis, rather than a one size fits all approach. We anticipate that the microbial screening of the IVF culture system will be most useful for couples with idiopathic infertility or repeated adverse IVF outcomes.

Microorganisms within the upper genital tract appear to be the norm, rather than the exception, despite the central dogma that the sterile upper genital tract is separated from the non-sterile lower genital tract by the cervix and cervical mucus plug. Women undergoing IVF are exposed to frequent iatrogenic procedures which can disrupt the surface microbiota including vaginal ultrasound scans, trans-vaginal oocyte retrieval and embryo transfer. These procedures rarely result in overt infection; however, they do have the potential to result in localized inflammation and inoculation of the IVF culture system with potential pathogens (Bennett, 1993, Bergh, 1992, Dicker, 1993, Tureck, 1993). It would appear that systemic antimicrobial treatment in most cases would be unnecessary and even detrimental to the IVF cycle, simply serving to enhance the possibility of opportunistic infection in either the male partner or the female partner. The over-prescription of antimicrobials may also create an environmental pressure leading to selection for resistant microorganisms. Microorganisms easily contaminate the IVF culture system. We propose three major areas for consideration in reducing the impact of microbial contamination on the IVF culture system: (1) changes to IVF culture techniques, (2) screening of patient samples collected during IVF treatment cycles, and (3) further research (Table II). Further research should focus on reducing the impact of these microorganisms in couples where there is no other explanation for IVF failure. ■

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