Contamination of Human IVF Cultures by Microorganisms: A Review

Kimball O. Pomeroy, PhD, HCLD
Laboratory Director, Arizona Reproductive Medicine Specialists
Contact: kpomeroy@arizonarms.com

Introduction

The IVF culture system is not a sterile system. Not only is the environment we work in full of bacteria, fungi and viruses, but our patient's bodies (follicular aspirates, semen and the vaginal and cervical regions for egg retrieval and embryo transfer) also contain microbes. Regardless of the most meticulous care taken to provide a sterile environment, when eggs, sperm and embryos are cultured, these cultures contain microbes - microbes that can result in the overt contamination of embryo culture media. The demise of valuable embryos due to contamination will, at the least, result in a patient with a wasted cycle and zero chance for pregnancy.

One of the most difficult circumstances clinical embryologists face is the discovery that after weeks of treatment, thousands of dollars of expense and hundreds of hours of invested time, a patient's embryos are contaminated with microorganisms and are unviable. If the contaminate is bacterial, the embryos may look dark and atretic. In most cases of bacterial contamination, the embryos are non-viable. Almost immediately, embryologists assume the source of contamination is due to poor sterile technique – they have inadvertently contaminated the culture medium or a sperm preparation. Although this is a possibility that should be investigated, most cases of contamination are probably due to microorganisms from the patient.

A second potential negative effect when embryo culture media are contaminated is the transmission of microorganisms into the patient which could result in either decreased endometrial receptivity or a clinical infection of the patient. To begin to understand contamination and reduce its occurrence, we need to know the sources of the contamination, how often it occurs and finally, why these contaminations occur.

The Non-Sterile Laboratory

The air that surrounds our bodies is loaded with bacteria, fungi and viruses. In fact, our body contains 10 times more bacterial cells than human cells. Our skin is colonized with plaques of Staphylococcus epidermides and corynebactria. Our skin literally snows bacteria. Every month we get a new dermis as 30,000 to 40,000 skin cells fall off every minute. These skin cells make up much of the dust that is swept up in the laboratory. The air is also a highway for bacteria. The outside air contains 10 to 100 colony forming units per cubic meter and the inside air contains 10 times more CFUs than the outside air. Our shedding skin is probably responsible for this increased bacterial load of inside air. Walls and ceilings are rarely heavily contaminated and usually contain 2-5 colonies per 25 cm2. Floors are more commonly contaminated with 380 colonies per 25 cm2. Most of this contamination is in the form of skin flora from occupants. Simple cleaning can reduce this load by 80% and the use of a disinfectant can reduce it to 95%. We work in a contaminated environment and our skin is often the source of the microbes found in our laboratories.

The most common bacteria cultured from the skin are Staphylococcus epidermides, Staphylococcus aureus, Acinetobacter, Klebsiella and other enterobacters, like E. coli. An experiment conducted in 2008 by Dalstrom et al demonstrates just how contaminated our working environment might be. Forty-five sterile trays were placed into operating rooms with differing levels of traffic. After opening the trays, a sterile drape was placed on a few of the trays while the remainder were left open in the clean air environemnt of the OR. Ten percent of the trays became contaminated almost immediately after opening and after 4 hours 30% were contaminated by bacteria or fungi. Only the drape-covered trays remained sterile.

Laboratories take many precautions to ensure that contamination of medium does not occur. Laboratories are usually equipped with HEPA filtration to reduce

the presence of airborne particles that might harbor microorganisms. They are engineered with a positive air-flow to ensure that the cleanest air flows into the laboratory and dirtier air is unable to leak into the laboratory. Even if perchance bacteria get into culture media, almost all of the media used in IVF contains antibiotics to inhibit bacterial growth. In the past, these antibiotics were often penicillin and streptomycin, but because of the short half-life of penicillin at 37 C less than 2 hours), most media now contains a stable form of the aminoglycoside, gentamicin.

Embryologists have a difficult task of trying to maintain a sterile environment while at the same time trying to protect the embryos from a toxic or inhospitable environment. Some of the methods we employ to keep the environment aseptic may exacerbate other problems resulting in lower embryo viability. For example, a hood used to maintain a sterile air flow may cause inadvertent changes in the osmolality of small micro drops, especially in a dry climate. This increased osmolality might result in osmotic stress either decreasing viability of the embryos or even making some degenerate. The improper use of hoods may even increase the chances for contamination if there are non-sterile items in the hood interrupting the flow of sterile air and causing that contaminated air to flow across sterile dishes or drops.

Survey on the Incidence Of Contamination

In all studies on the incidences of microbial contamination it is important to remember that the incidences of microbial contamination depend on the methods used to find microbes. Retrospective studies most likely only turn up overt contamination events where flocculants are observed at low magnification (100 to 400X) during embryo culture. The methods used for detection also will result in different incidences. In the past, selective culture media has been used to identify bacteria. Within the last few years, PCR-based methods have been used which are much more sensitive. PCR-based methodsto are more accurate than routine culture methods in the identification of microorganisms. In other words, what one finds is often determined by the sensitivity of the tools that are used.

To better understand the sources and incidence of microbial contamination of embryo cultures, we invited several IVF laboratories to participate in an online survey. Thirty-two laboratory directors with 97% of them having over 10 years of experience participated in this survey. The survey revealed that the most common sources of contamination were semen (32%) and improper sterile technique (23%). Forty-nine percent of the the time the contaminant was identified as a bacterium and 51% of the time as a fungi (yeast or mold). The most common species identified were E. coli, Aspergilllus, C. albicans and gram negative cocci. It should be remembered that probably most of these incidences of contamination occurred while penicillin and streptomycin were used as antibiotics in culture media and so this list probably best describes those bacteria that can aquire antibiotic resistance to these particular antibiotics. Semen, technician contamination and oil were the most often quoted sources of these contaminations.

Retrospective Studies

Several studies have looked at the incidence of microbial contamination during IVF treatment. Kastrop et al. (2007) examined almost 14,000 IVF cycles at their clinic and found that 0.7% of IVF cycles had microbial contamination. Interestingly, none occurred in 2962 ICSI cycles, inferring that the most likely source of the contamination was something that was unique to standard IVF when compared to ICSI. The most likely candidate would probably be the sperm preparation. The most common contaminants found were E. coli (58.9%) and Candida species (25.3%). When bacteria was identified as the source of contamination, 91% of the time the bacteria was resistant to penicillin or streptomycin. Since moving to Gentamicin in 2005, they reported no incidences of bacterial contamination, only fungal. Of the 70 bacterial strains they isolated from contaminated culture media, all were subsequently found to be sensitive to Gentamicin. Kastrop et al observed compromised embryo quality in all media with bacterial contamination, but yeast appeared to have no affect on embryo quality.

Another study (Ben-Chetrit et al, 1996) examined 729 IVF cycles in where 5 cases of yeast contamination (incidence = 0.7%) were found. After extensive rinsing of the embryos in these five cases, one to three embryos were transferred into each patient and all

five became pregnant. Ben-Chetrit et al hypothesize that the yeast contamination may have come during follicular aspiration where yeast are a common flora of the vagina and that the yeast may have had a beneficial effect on the embryos by decreasing the concentration of oxidative free radicals, either by decreasing oxygen tension or via glutathione reductase activity of yeast.

Prospective Studies

Cottell et al (1996) performed a prospective study to identify the incidence and sources of fungal and bacterial contamination during 30 IVF cycles. They cultured swabs during different aspects of the IVF process in 30 IVF cycles. They found microorganisms in half of the IVF cycles they examined. This indicates a high level of contamination despite a fairly insensitive method to determine contamination. Despite this, they saw no overt growth during any of the embryos cultures. Of the 15 cases with bacterial growth, 4 semen specimens (27%), all follicular aspirates (100%) and two of the fertilization cultures (13%) were found to be contaminated. Microbes were not isolated from any of the long-term embryo cultures. The most common organisms found were Mycoplasma hominis, Stapylococcus epidermides and Diptheroids. This study shows that the microbes are present at a high incidence in the IVF cultures, even during fertilization. The most common source appears to be from the follicular aspirates. There are several explanations for the low incidence of contaminated cultures, despite the high incidence of contamination. It is possible that a combination of events results in a low incidence of overt bacterial contamination of embryo cultures. This could include low numbers of organisms especially after the many dilutions with media that occur during processing, slow growth of organisms, the presence of antibiotics, and the embryo culture media may be suboptimal for many bacteria.

Semen As A Source Of Microbial Contamination

One potential source of contamination during embryo culture is semen. Semen is not a sterile fluid and has been shown to often contain microorganisms. Cottell et al (1997) in a prospective study investigated the incidence of microbial contamination from 140 semen specimens. They found that 63% of all semen specimens were contaminated with bacteria. The most common organism found was Staphylococcus epidermidis (29%), one of the most common bacteria found on skin. They also isolated nonhemolytic streptococci (30%), diptheroids (14%), b-streptococci (12.1%), Streptococcus viridans (10%) and E. coli (6.4%). Very few anaerobes were identified but mycoplasmas were found in about 17% of the semen specimens.They found that they could reduce this incidence of contamination by using antibiotics during semen processing. After collection in antibiotic-containing media and a sperm wash and swim up, only 5% of specimens remained positive for bacterial contamination.

In another study using more sensitive PCR detection methods, bacteria were detected in 65% of the men (Kiessling et al, 2008). This was one of the first attempts at surveying semen using the new molecular techniques. The most common bacteria were gram-positive anerobic cocci, followed by *Corynebacterium* spp., *Staphylococcus*, *Lactobacillus* and *Streptococcus*. Two important conclusions should be noted from this study: 1) Leukocytospermia is not a reliable indicator of the presence of bacteria in the semen and 2) the most common bacteria found in this study were not the same organisms reported by bacterial culture studies, most likely due to the difficulty of culturing these bacteria. This emphasizes the point that what have been found in past studies may not relate to what are actually growing in the sampled tissues or fluids, but were affected by the limitations of the older technologies. In other words, what was detected was not necessarily what was there, but what that particular technique was able to detect.

Chlamydia is a common contaminate of semen. In one study examining men at an infertility clinic, 13% contained detectable levels of *C. trachomatis* DNA in their semen. This study further showed that gradient centrifugation is inefficient in removing this bacterium from semen. This may be due to the fact that C. trachomatis can bind sperm and form inclusion bodies in sperm. Semen has also been shown to contain endotoxins.

Viruses are also found in semen and have the potential to impair embryo culture, implantation and pregnancy. HIV-1 has been shown to be present in the semen of AIDS patients and the virus has been shown

to bind spermatozoa and transfer HIV-like particles to human oocytes. Herpes viruses have also been found in human semen. One study of 172 men found herpes virus in 83% of their semen (Neofytou et al, 2009). This included HHV6, EBV and CMV. We do not yet know though whether the presence of these viruses can affect embryo culture, but we do know that they can alter sperm motility (Lai et al, 1997).

Follicular Aspirates

Another potential source of contamination are the follicular aspirates from which oocytes are obtained. To obtain these aspirates, a relatively large gauge needle is passed through the vagina and into the ovary. The vagina is a great source of bacteria and fungi. One of the most common microorganisms found in the vagina are lactobaccili (100% of women without vaginosis). Other bacteria commonly found in the vagina of healthy women include *Staphylococcus epidermides*, *Clostridium perfringens* and ureaplasma. It is possible that the follicular fluid could become contaminated during the passage of the needle through the vagina.

One recent unpublished study (Knox, personal communication) may indicate that the follicle aspirate is the primary source of bacterial and/or fungal contaminates in IVF culture. Knox et al studied the initial aspirates from 144 follicular aspirations of women undergoing IVF. They cultured samples from each initial aspirate and determined if any microorganisms were present and the type of organism. Surprisingly, they found microorganisms in 143 of the 144 aspirates. In an attempt to determine if the organisms came from the vagina or the ovary, they compared the species from the vagina to those from the follicular aspirates. Thirty-five percent of follicular aspirates contained organisms that were not found in the vaginal cultures. When this occurred, pregnancy rates were about 50% lower than those cases where the same organisms were isolated from both the ovary and the vagina. These investigators also noted that unique ovarian microorganisms were more common in patients that had prior IVF cycles – indicating that prior follicular aspiration might serve to inoculate the ovaries with bacteria.

Embryo Transfer

Bacterial contamination may not only affect embryo quality, but there are also reports of reduced pregnancy rates when bacteria are isolated from the embryo transfer catheter used during a trial transfer (Fanchin et al, 1998) just prior to embryo placement, or from the catheter used during the actual transfer (Egbase et al, 1996). Another study (Selman et al, 2007) demonstrated decreased pregnancy rates when either Entrobacteriacea or Staphylococcus were isolated from the fundus of the vagina, the cervix or the tip of the embryo transfer catheter. There are three possible mechanisms for these decreased pregnancy rate: 1) The presence of high levels of bacteria on the cervix may be due to chronic endometritis which may result in a lowered endometrial receptivity. 2) The transfer procedure may carry bacteria from the cervix into the uterus where it may alter the endometrium and hinder implantation. 3) The process of embryo transfer may contaminate the embryo with bacteria resulting in a direct negative effect on the embryo. It is not necessary for bacteria to actually bind (infect) a cell to have a negative effect. Cultures in which bacteria have grown can contain many toxic substances including endotoxins, alpha-hemolysin, Shiga-like and other lipopolysaccharides and pedtidoglycans. Endotoxin, the lipopolysaccharide portion of the cell wall of gram-negative bacteria has been found to influence embryo culture. It can cause fragmentation, blebbing and reduce pregnancy rates (Zarutskie et al, 1992; Fischel et al, 1988). Both the vagina and semen have been shown to be sources of endotoxin contamination (Zarutskie, et al, 1992) as well as water used to manufacture media.

Recently, the effects of E. coli on spermatozoa have shown that binding and infectivity of cells are not necessary for bacteria to have a negative effect on cells. (Schulz et al., 2010 Effect of Escherishia coli and its soluble factors on mitochondrial membrane potential, phosphatidylserine translocation, viability and motility of human spermatozoa, Fertil Steril 94:619-623.) It has been hypothesized that alpha-hemolysin, a calcium-dependent cytolytic toxin secreted by E. coli might be partially responsible for toxic affects on spermatozoa cultured in media conditioned by bacteria. Alpha-hemolysin does not require a receptor for cell binding and can insert itself into the cell membrane forming pores that permit a rapid release of potassium and an influx of calcium, manitol and sucrose, which leads to cell destruction by osmotic lysis (Wai SN et al, 2003 Cell 115:25-35 Vessicle-mediated export and assembly of pore-forming oligomers of the enterobacterial ClyA cytotoxin). Other potential E. coli toxins that might be responsible for cell death by E. coli supernatants include Shiga-like, lipopolysaccharides and pedtidoglycan fragments.

Most of the studies mentioned above rely on differential culturing to determine the presence of bacteria. This method is much less reliable than the newer PCR-based methods. One cannot assume that just because one does not seen any obvious microbial contaminants that they are not present in embryo cultures, or that they do not affect IVF outcomes. Microbes may be present at levels that are undetectable through the low-powered microscopes used for routine embryo culture. Some microbes may be in low concentrations, in poor culture environments where the growth of these microbes is slow enough to seldom reach levels that result in visible flocculent. It is possible that what we often attribute to patient variability in embryo culture may be in part due to due to variation of microbial flora in each patient and subsequent non-overt contamination. It is likely that many IVF cultures are contaminated with bacteria or viruses that affect the culture of the embryos, but do not produce any overt signs such as flocculants or necrosis of the embryos. A similar condition was reported in cell cultures several years ago with mycoplasm and tissue culture.

Mycoplasma

In the early 1960s, it was discovered that most cell lines used in research had been contaminated by the bacterium Mycoplasma (I Macpherson, Mycoplasmas in tissue culture. J. Cell Sci.1:145-168, 1966). This bacterium affected many properties of cell lines used in research. For years, somatic cell lines were propagated without knowledge that these lines were contaminated with mycoplasma. It has been estimated that mycoplasma may contaminate as much as 40% of all tissue cultures. The effects of mycoplasma on cells include decreased cell growth, pH shifts, depletion of cell substrates, induction of oxidative stress, ammonia production,

secretion of other harmful metabolites, and modification of DNA, RNA and protein synthesis. Certain strains of mycoplasma preferentially deplete specific amino acids including arginine, glutamine and glutamic acid (Smith, PF. Amino acid metabolism of PPLO. Ann. N.Y. Acad. Sci 79:543-550. 1960). Many studies using infected cells in the fields of virology, biochemistry, oncology and immunology resulted in the misinterpretation of data - attributes attributed to treatments may have been due to mycoplasma contamination.

Mycoplasma is a difficult organism to remove from cell cultures. Penicillin does not affect mycoplasma, as these very small (0.15 to 0.8 micron) bacteria do not have a cell wall. Mycopasma are susceptible to gentamicin, but at a concentration 20 times that normally found in culture medium. Mycoplasma can outnumber a cell 1000 to 1 and their small size allows them to pass through a 0.22 micron filter. (Most filter sterilization in IVF uses 0.22 u filters which allow this small malleable bacterium to pass through the filter. Mycoplasma has been implicated in infertility, stillbirths, prematurity, meningitis and is a common contaminant found in semen. This is just one bacterium that could have a potential role in influencing embryo culture and pregnancy rates (not to mention infertility), that shows very little overt signs, whose influence has not been researched. The affects of mycoplasma on embryos could occur without any other overt affects, or they might be accompanied by embryo fragmentation, blebbing, poor embryo quality or other more overt signs (necrosis).

Fungi And Viruses

Besides bacteria, fungi are often found contaminating IVF cultures. Unlike bacteria though where embryos are often necrotic, embryos often appear to tolerate co-culture with fungi. The source of the contaminating fungi most likely is from the follicular aspiration or it could be an airborne contaminate. One reason why fungal contamination might be common in human IVF is that culture medium routinely does not contain anti-fungal agents. What is surprising is that it does not occur more often, but this may be due to the fact the embryo culture environment is not conducive to most fungal growth. In one retrospective study (3), yeast contaminated the IVF cultures of 5 patients (of 729). Embryo quality was not compromised and after thorough washing the embryos were transferred into the patients. All 5 patients conceived.

Although we know quite a bit how viruses affect humans, we know very little about the role of viruses as toxic contaminates in IVF cultures. No prospective studies have been conducted to look at the incidence of viral contamination during IVF. Viral contaminates could enter the IVF system from follicular aspirates, semen or the laboratory environment. Semen has been shown to be routinely contaminated with viruses. One study (5) found that 83.1% of men at an infertility clinic had herpes virus identified in their semen. The most common types were HHV-6 (66.8%), CMV (56.9%) and EBV (40.6%). Just as mycoplasma contamination of cell lines can affect the properties (including viability) of cultured cells, so can viruses. For example, rodent parvoviruses can infect rodent tissue cultures and modify the behavior of these cells (Nicklas, W, Kraft, V, Meyer, B (1993) Contamination of transplantable tumors, cell lines and monolonal antibodies with rodent viruses. Lab. Anim. Sci. 43:296-300.)

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Conclusions

IVF does not occur in a sterile environment. Despite the most fastidious environmental engineering and sterile techniques, the most we can hope for is to reduce the levels of microorganism contamination in our laboratories and in our embryo cultures. The key cells we use to produce embryos, sperm and eggs, come from sources that are most likely contaminated with viruses, fungi and bacteria. There is no detailed, definitive survey on the incidence of these contamination events nor on the influence these microorganisms might play in the culture of human embryos, nor has a complete prospective microbiological survey of the tissues and fluids involved in IVF been performed using new, sensitive PCR-based methods.

The cost of microbial contamination to our society can be calculated with estimates of overt contamination, the number of IVF cycles done per year and the cost of the IVF procedure. Two studies have indicated that the incidence of overt bacterial contamination is about 0.7%. Approximately 90,000 IVF cycles were performed in the United States in 2008 (SART National Registry). This means that about 630 cycles resulted in bacterial contamination where, in most cases, no embryos were transferred. The estimated cost to these patients for these contaminations would be about $10,000 per patient (this does not include the amount of wasted time and other resources) - resulting in an overall cost of about $6.3 million. This enormous cost does not include the costs to those patients that did not get pregnant due to potentially non-overt contamination of embryo cultures that might have had reduced the pregnancy rates. For such a potentially pervasive problem it is imperative that we understand the effects of these microorganisms on human embryo culture and that we develop methods to decrease these effects of microbial contamination on human embryo culture. ■

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